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BIOGENEX LABORATORIES, INC.

UNITED STATES DISTRICT COURT

NORTHERN DISTRICT OF CALIFORNIA

SAN JOSE DIVISION

BIOGENEX LABORATORIES, INC., a California corporation,

Plaintiff

vs.

VENTANA MEDICAL SYSTEMS, INC., an Arizona corporation,

Defendant.

C03 03916 JCS

COMPLAINT FOR PATENT INFRINGEMENT

(U.S. PATENT NOS. 5,578,452, 5,244,787 & 6,451,551)

JURY TRIAL DEMANDED

Plaintiff, BIOGENEX LABORATORIES, INC., ("BioGenex") alleges as follows:

THE PARTIES

1. Plaintiff BioGenex Laboratories, Inc. ("BioGenex") is a corporation existing under the laws of the State of California with its principal place of business at 4600 Norris Canyon Road, San Ramon, California 94583.

2. On information and belief, defendant Ventana Medical Systems, Inc. ("Ventana") is a corporation existing under the laws of the State of Arizona with

its principal place of business at 1910 Innovation Park Dr., Tucson, Arizona 85737. On information and belief, Ventana is doing business within the State of California and this District.

3. This Court has personal jurisdiction over the Defendant pursuant to Cal. Code Civ. Proc. Section 410.10. On information and belief, Ventana solicits sales, sells products and attends trade shows within the District of California, has continuous and substantial contacts with the State of California and is the major supplier of automated systems for Immunohistochemistry (IHC), In Situ Hybridization (ISH), and Special Stains with offices in North America, France, Japan and Australia. Ventana has committed and continues to commit acts of patent infringement in this District, the State of California and elsewhere in the United States.

4. This action is brought under the Patent Laws of the United States, 35 U.S.C. §§ 271 and 281. This Court has original and exclusive jurisdiction over this controversy under 28 U.S.C. §§ 1338(a) and 1331.

VENUE

5. Venue is proper in this judicial district pursuant to 28 U.S.C. § 1391(b) and (c) and 28 U.S.C. § 1400.

INTRADISTRICT ASSIGNMENT

6. For purpose of Local Rule 3-5(b), this action arises in Contra Costa County, where BioGenex is located and where a substantial part of the actions and infringements described below were directed.

FACTUAL BACKGROUND

7. BioGenex develops, manufactures and distributes molecular and cellular diagnostic systems. Among other things, BioGenex sells products to laboratories that perform Immunohistochemistry (IHC) testing. IHC testing utilizes antibody molecules to identify and localize specific antigens (such as cancer markers) in these tissue specimens.

8. BioGenex has long been in the forefront of the technological development of new methods and products for performing immunostaining, an IHC procedure used widely in clinical and research facilities. It has pioneered the field of antigen retrieval for pretreating tissue samples for more reliable IHC testing.

9. On November 26, 1996, United States Patent No. 5,578,452 (the "'452 patent") disclosing and claiming an invention entitled ENHANCEMENT OF IMMUNOCHEMICAL STAINING IN ALDEHYDE-FIXED TISSUES was duly and legally issued from an application originally filed on April 7, 1994 in the name of inventors Shan-Rong Shi, Atul K. Tandon, Krishan L. Kalra, Nagesh Malhotra, Sheng-Hui Su and Cheng-Zhi Yu. A true and correct copy of the '452 patent is attached hereto as Exhibit A.

10. On September 14, 1993, United States Patent No. 5,244,787 (the "'787 patent") disclosing and claiming an invention entitled ANTIGEN RETRIEVAL IN FORMAL IN FIXED TISSUES USING MICROWAVE ENERGY was duly and legally issued from an application originally filed on January 31, 1991 in the name of inventors Marc E. Key, Shan-Rong Shi and

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Krishan L. Kalra. A true and correct copy of the '787 patent is attached hereto as Exhibit B.

11. On September 17, 2002, United States Patent No. 6,451,551 (the "'551 patent") disclosing and claiming an invention entitled RELEASING EMBEDDING MEDIA FROM TISSUE SPECIMENS was duly and legally issued from an application originally filed on May 16, 2000 in the name of inventors Guangrong Zhan, Krishan L. Kalra, Sheng-Hui Su and Taiying Chen. A true and correct copy of the '551 patent is attached hereto as Exhibit C.

12. BioGenex is the assignee and owner of all right, title and interest in and to the '452 patent, the '787 patent and the '551 patent and has the right to bring this suit for damages and injunctive relief.

13. Defendant has been notified verbally that the activities alleged herein constitute infringement of the '452 patent, the '787 patent and the '551 patent.

FIRST CLAIM FOR RELIEF

(Direct Patent Infringement - 35 U.S.C. § 271(a) the '452 Patent and the '551 Patent)

14. BioGenex realleges and incorporates by reference Paragraphs 1 through 13 inclusive.

15. On information and belief, Ventana manufactures, sells and/or offers to sell a product or products (a) for use in methods to enhance the immunological staining of aldehyde-fixed tissues as disclosed and claimed in the '452 patent and (b) to release embedding media from tissue specimens as disclosed and claimed in the '551 patent.

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16. On information and belief, Ventana has offered to and has demonstrated the use of its products in the Northern District of California and elsewhere in a manner that directly infringes the '452 patent and the '551 patent.

17. On information and belief, Ventana continues to infringe the '452 patent and the '551 patent.

18. BioGenex has been irreparably harmed by said acts of infringement, and will continue to be harmed unless Ventana's further acts of infringement are restrained by order of the Court. BioGenex has no adequate remedy at law.

19. As a result of Ventana's activities and acts of infringement, BioGenex has suffered and will continue to suffer damages in an amount to be proven at trial.

SECOND CLAIM FOR RELIEF

(Inducement of Patent Infringement - 35 U.S.C. § 271(b) of the '452 Patent, the '787 Patent and the '551 Patent)

20. BioGenex realleges and incorporates by reference Paragraphs 1 through 19 inclusive.

21. On information and belief, Ventana, with intent to induce infringement of the '452 patent, the '787 patent and the '551 patent by others, has induced and is inducing such infringement, including but not limited to, through the marketing, sale and instructions for use of its Cell Conditioning solution.

22. On information and belief, Ventana provides written and oral instructions to actual and prospective customers to use the Cell Conditioning

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solution (a) to enhance the immunological staining of aldehyde-fixed tissue as disclosed and claimed in the '452 patent, (b) for immunological staining of formalin-fixed tissues as disclosed and claimed in the '787 patent and (c) to release embedding media from tissue specimens as disclosed and claimed in the '551 patent.

23. On information and belief, use of the Cell Conditioning solution as instructed by Ventana will result in the infringement of the '452 patent, the '787 patent and the '551 patent.

24. On information and belief, defendant continues to induce the infringement of the '452 patent, the '787 patent and the '551 patent.

25. BioGenex has been irreparably harmed by said acts of inducement of infringement, and will continue to be harmed unless Ventana's further acts of infringement are restrained by order of the Court. BioGenex has no adequate remedy at law.

26. As a result of Ventana's activities and acts of inducement of infringement, BioGenex has suffered and will continue to suffer damages in an amount to be proven at trial.

THIRD CLAIM FOR RELIEF

(Contributory Infringement - 35 U.S.C. § 271(c) of the '452 Patent and the '551 Patent)

27. BioGenex realleges and incorporates herein by reference Paragraphs 1 through 26, inclusive.

28. On information and belief, Ventana's Cell Conditioning solution is used by customers (a) to enhance the immunological staining of aldehyde-fixed tissues as disclosed and claimed in the '452 patent and (b) to release embedding media from tissue specimens as disclosed and claimed in the '551 patent.

29. On information and belief, the Cell Conditioning solution is specially adapted so that when used as instructed by Ventana (a) to enhance the immunological staining of aldehyde-fixed tissues it will infringe the '452 patent and (b) to release embedding media from tissue specimens it will infringe the '551 patent.

30. On information and belief, Ventana knows that the Cell Conditioning solution is specially adapted for use in the methods described and claimed in the '452 patent and the '551 patent.

31. On information and belief, the Cell Conditioning solution is not a staple article or commodity of commerce suitable for a substantial noninfringement use.

32. On information and belief, Ventana continues to contributorily infringe the '452 patent and the '551 patent.

33. BioGenex has been irreparably harmed by said acts of contributory infringement, and will continue to be harmed unless Ventana's further acts of contributory infringement are restrained by order of the Court. BioGenex has no adequate remedy at law.

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34. As a result of Ventana's activities and acts of infringement, BioGenex has suffered and will continue to suffer damages in an amount to be proven at trial.

PRAYER FOR RELIEF

WHEREFORE, BioGenex prays that this Court enter a judgment and decree:

1. A declaration that defendant has and is infringing the '452 patent;

2. A declaration that defendant has and is infringing the '787 patent;

3. A declaration that defendant has and is infringing the '551 patent;

4. A permanent injunction enjoining defendant, its officers, directors, employees, agents and attorneys and all persons in active concert or participation with them from infringing the '452 patent;

5. A permanent injunction enjoining defendant, its officers, directors, employees, agents and attorneys and all persons in active concert or participation with them from infringing the '787 patent;

6. A permanent injunction enjoining defendant, its officers, directors, employees, agents and attorneys and all persons in active concert or participation with them from infringing the '551 patent;

7. A permanent injunction enjoining defendant, its officers, agents, servants, employees, and attorneys, and those persons in active concert or participation with them, from selling to others any products developed in whole or

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in part by defendant for enhanced immunostaining that when used results in the infringement of the '452 patent;

8. A permanent injunction enjoining defendant, its officers, agents, servants, employees, and attorneys, and those persons in active concert or participation with them, from selling to others any products developed in whole or in part by defendant that when used results in the infringement of the '787 patent;

9. A permanent injunction enjoining defendant, its officers, agents, servants, employees, and attorneys, and those persons in active concert or participation with them, from selling to others any products developed in whole or in part by defendant that when used results in the infringement of the '551 patent;

10. A permanent injunction requiring defendant to cease the manufacture of all products developed in whole or in part by defendant for performing enhanced immunostaining that when used results in the infringement of the '452 patent;

11. A permanent injunction requiring defendant to cease the manufacture of all products developed in whole or in part by defendant that when used results in the infringement of the '787 patent;

12. A permanent injunction requiring defendant to cease the manufacture of all products developed in whole or in part by defendant that when used results in the infringement of the '551 patent;

13. A permanent injunction requiring the impoundment and destruction of all products developed in whole or in part by defendant which when used violate the '452 patent;

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14. A permanent injunction requiring the impoundment and destruction of all products developed in whole or in part by defendant which when used violate the '787 patent;

15. A permanent injunction requiring the impoundment and destruction of all products developed in whole or in part by defendant which when used violate the '551 patent;

16. Awarding BioGenex damages, together with prejudgment interest, based on defendant's infringement of the '452 patent and trebling the same pursuant to 35 U.S.C. § 284 for the willful, wanton and deliberate nature of such infringement;

17. Awarding BioGenex damages, together with prejudgment interest, based on defendant's infringement of the '787 patent and trebling the same pursuant to 35 U.S.C. § 284 for the willful, wanton and deliberate nature of such infringement;

18. Awarding BioGenex damages, together with prejudgment interest, based on defendant's infringement of the '551 patent and trebling the same pursuant to 35 U.S.C. § 284 for the willful, wanton and deliberate nature of such infringement;

19. Awarding BioGenex its costs and reasonable attorneys' fees pursuant to 35 U.S.C. § 285;

20. That defendant be directed to file with this Court and serve on Plaintiff within thirty days after the service of an injunction, a report in writing and

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under oath setting forth in detail the manner and form in which defendant has complied with the injunction; and

21. Granting BioGenex such other and further relief as this Court deems proper and just.

Respectfully submitted,

Dated: 08/22/03

By: _____

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DEMAND FOR JURY TRIAL

Pursuant to Rule 38 Fed. R. Civ. Proc., Plaintiff Biogenex Laboratories, Inc. hereby demands a trial by jury.

DATED: August 22, 2003

Respectfully submitted,

By: _____

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EXHIBIT A

US005578452A

United States Patent [19]

Shi et al.

[11] Patent Number: 5,578,452

[45] Date of Patent: Nov. 26, 1996

[54] ENHANCEMENT OF IMMUNOCHEMICAL STAINING IN ALDEHYDE-FIXED TISSUES

[75] Inventors: **Shan-Rong Shi**, Los Angeles; **Atul K. Tandon**, Fremont; **Krishan L. Kalra**, Danville; **Nagesh Malhotra**, Los Angeles; **Sheng-Hui Su**, San Ramon; **Cheng-Zhi Yu**, Pleasant Hill, all of Calif.

[73] Assignee: **Biogenex Laboratories**, San Ramon, Calif.

[21] Appl. No.: 211,595

[22] PCT Filed: Aug. 12, 1993

[86] PCT No.: PCT/US93/07550

§ 371 Date: Apr. 7, 1994

§ 102(e) Date: Apr. 7, 1994

[87] PCT Pub. No.: WO94/04906

PCT Pub. Date: Mar. 3, 1994

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 928,962, Aug. 12, 1992, abandoned, which is a continuation-in-part of Ser. No. 821,931, Jan. 16, 1992, abandoned.

[51] **Int. Cl.**[6] **G01N 33/567**

[52] **U.S. Cl.** **435/7.21**; 435/7.95; 435/40.52; 436/63; 436/175

[58] **Field of Search** 436/175, 63, 518, 436/528; 435/7.95, 7.21

[56] **References Cited**

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March, (1968) "Advanced Organic Chemistry," particularly at 333, 424, 670–672.

Mayers, *J. Clin. Pathol.* (1970) 28:273.

Hopwood et al., *Histochem. J.* (1984) 16:1171.

Battifora and Kopinski, *J. Histochem. Cytochem.* (1986) 34:1095–1100.

Huang, et al., *J. Lab Invest.* (1976) 35:383–390.

Leong, et al., *J. Pathology* (1988) 156:275–282.

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Carey et al., (1983) *Advanced Organic Chemistry*, 2nd ed. 58–62.

Fraenkel–Conrat et al., (1948) "Reaction of Formaldehyde with Proteins (IV) Cross–linking of Amino Groups with Phenol, Imidazole, or Indole Groups" *J. Biol. Chem.*, 174:827–843.

Primary Examiner—Mary E. Ceperley

Attorney, Agent, or Firm—Gray Cary Ware & Freidenrich

[57] **ABSTRACT**

A method for restoring immunoreactivity of a tissue, particularly decalcified tissue, fixed width an aldehyde fixative agent and embedded in an embedding medium, usually comprising celloidin, the method comprising the steps of contacting the tissue with an aldehyde releasing reagent solution comprising a solvent and an aldehyde releasing reagent and removing aldehyde released by the aldehyde releasing reagent from contact with the tissue by reacting the aldehyde in a substantially irreversible manner to form a non-aldehyde derivative, and removing excess base from the tissue. A preferred solution for celloidin-embedded decalcified tissue comprises methanolic sodium hydroxide at about one-third saturation.

15 Claims, No Drawings

ENHANCEMENT OF IMMUNOCHEMICAL STAINING IN ALDEHYDE-FIXED TISSUES

RELATED APPLICATION DATA

This application is a continuation-in-part of application Ser. No. 07/928,962, filed Aug. 12, 1992, now abandoned, which in turn is a continuation-in-part of application Ser. No. 07/821,931, filed Jan. 16, 1992, now abandoned.

TECHNICAL FIELD

The invention concerns immunohistochemical staining of aldehyde-fixed and embedded tissue sections.

BACKGROUND

Tissue sections obtained from clinical specimens or animal experimentation frequently are fixed, embedded, and stored in a form suitable for later examination by light microscopy. Immunological reagents, especially monoclonal antibody reagents, currently permit examination of at least certain of these fixed tissue samples for the presence of particular antigenic compounds. Antigens of interest may be associated with a disease process or pathology, or may identify a particular cell type or tissue. In the case of recently prepared biopsy and autopsy samples, such immunohistochemical analyses are of immediate diagnostic value.

However, immunohistochemical analyses of tissue specimens have been hampered because of antigenic loss during specimen fixation. Traditional fixation methods frequently have employed aldehyde fixatives, which fix the tissue by causing cross-linking reactions within and between tissue proteins.

Two types of cross-linking reactions have been recognized. The first is a Schiff's base-type polymerization: formaldehyde condenses with the amino groups of the protein, resulting in the Schiff's base intermediate, which is capable of undergoing rapid polymerization leading to cross-linking of the proteins.

In the second type of reaction, called the Mannich reaction, the formaldehyde can react with both an amino group and an active hydrogen group, resulting in the formation of a Mannich base. Polymerization of the Mannich bases results in protein cross-linking.

Cross-links preserve tissue morphology and integrity, harden the tissue for slicing, and inhibit microbial attack. Unfortunately, the cross-linking process also causes loss of tissue antigenicity, a result which impedes the usefulness of immunological reagents on tissues fixed with aldehyde reagents such as formaldehyde. The chemistry of the cross-linking of amino acids and proteins by formaldehyde is described in Harlan and Feairheller, "Chemistry of the Cross-Linking of Collagen During Tanning," and Kelly, et al. "Cross-Linking of Amino Acids By Formaldehyde," (1976). The role of Mannich-type reactions in cross-linking of protein amino groups and aromatic amino acids with formaldehyde is discussed in Fraenkel-Conrat, et al., *J. Biol. Chem.* (1947) 168:99–118, and Fraenkel-Conrat and Olcott, *J. Biol. Chem.* (1948) 174:827–843. Further discussions of aldehyde cross-linking reactions are found in Fox, *J. Histochem. Cytochem.* (1985) 33:845–855; Jones, "Reactions of aldehyde with unsaturated fatty acids during histological fixation," in *Fixation in Histochemistry*, P. J. Stoward, ed. (1973); and Kunkel et al., *Mol. Cell. Biochem.* (1981) 34:3. Mannich type reactions are described in general in March, "Advanced Organic Chemistry," particularly at 333,424, 670–672 (1968).

In an attempt to circumvent the disadvantages of aldehyde fixation, alternative fixation methods have been developed, such as microwave heating (Mayers, *J. Clin. Pathol.* (1970) 28:273; Hopwood et al., *Histochem. J.* (1984) 16:1171) and alcohol immersion (Battifora and Kopinski, *J. Histochem. Cytochem.* (1986) 34:1095). Despite some advantages of alternative fixation methods, they have not displaced aldehyde fixation in general use. Their limited acceptance may reflect drawbacks present in these alternative methods. For example, microwave heating lyses red cells and disrupts membrane lipids. Although ethanol fixation is reported to produce improved antigenicity of tissue samples, ethanol causes increased cellular shrinkage (Battifora and Kopinski, id.) Consequently methods for restoring antigenicity to aldehyde fixed tissues continue to be useful for specimens generated by current clinical practices.

In addition, methods for restoring antigenicity are useful because of the vast number of aldehyde fixed tissue samples already in collections. These stored tissue samples provide a rich reservoir of material for retrospective immunohistochemical examination. If a suitable method of subsequent immunohistochemical staining were available, newly generated immunohistochemical data could be combined with existing diagnostic results obtained from traditional investigations on the same tissues. Often clinical samples are saved for decades, so that the clinical outcome of the patient's underlying pathological process already is known. In the case of experimental tissues, such as those obtained from animals in toxicology testing, other measurements of pathology and toxicity in general already will have been performed and documented. In both cases, immunological analyses of the affected tissues could add important correlative information.

Because of the development of immunological reagents over the past decades, immunohistochemical analyses can now be performed that were impossible at the time many tissues were originally stored. In addition, new knowledge or hypotheses concerning the disease process may prompt reexamination of stored tissues. Immunohistochemical studies on stored tissue samples provide a relatively time- and cost-effective means for performing a clinical study on a statistically large sample population. Therefore the application of immunohistochemical analyses to routine clinically or experimentally derived embedded tissue sections is a matter of considerable interest.

Antigenic loss during aldehyde tissue fixation is due to chemical modification of the protein (not to physical removal of the antigen). Loss of immunoreactivity is believed to occur by two mechanisms. In the first mechanism, the fixative agent chemically modifies the reactive epitope, rendering it incapable of binding antibody. In the second mechanism, the fixative agent causes chemical cross-linking of the antigenic protein at sites outside the targeted epitope. Such cross-linking may be intramolecular or intermolecular, i.e., with involvement of nearby proteins. This cross-linking sterically hinders access of the antibody reagent to the reactive epitope.

The second mechanism, steric hindrance due to intraprotein or interprotein cross-linking outside the epitope of interest, has been reversed by protease digestion of formalin fixed tissues in order to remove the interfering cross-linked portions of proteins. This approach has been shown to improve immunostaining of keratins in foxmaldehyde fixed tissues (Battifora and Kopinski, *J. Histochem. Cytochem.*

(1986) 34:1095–1100). However, protease treatment actually degraded the staining of tissues fixed in alcohol, a fixative solution which does not cause protein cross-linking. Some improvement in immunostaining by proteolysis of formaldehyde fixed tissues for limited time periods has been shown for other antigens (Huang, et al., *J. Lab Invest.* (1976) 35:383–390.) In a study of immunostaining of 23 antigens of pathological interest, prior trypsinization was shown to give no improvement in immunostaining of formaldehyde fixed tissues except in the case of cytokeratins and desmin. For many antigens, enzyme digestion actually diminished antigen staining. (Leong, et al., *J. Pathology* (1988) 156:275–282.) These results support the hypothesis that aldehyde fixative-induced cross-linking of proteins diminishes immunostaining both by chemical modification of epitopes and by steric hindrance mediated by cross-linking. The mixed results obtained from treating fixed tissues with proteolytic enzymes are readily rationalized: although partial proteolysis decreases cross-linking and reduces steric hindrance, proteolysis also may cleave and remove the epitopes of interest. Moreover, proteolysis cannot reverse antigenic masking due to chemical modification of the epitope. By contrast, a procedure which could reverse the chemical cross-linking reaction produced by aldehyde fixatives has the potential to unmask antigens previously hidden by either mechanism.

A procedure for restoring antigenicity of formalin-fixed, paraffin embedded tissue sections by heating the tissue in a microwave in a heavy metal solution has been described in Shi, et al. *J. Histochemistry and Cytochemisty* 39(b): 741–48 (1991). This procedure provides enhanced immunostaining in approximately three-fourths of the samples tested. The described method is part of a process that involves the steps of tissue section deparaffinization and rehydration, brief treatment with aqueous peroxide to block endogenous peroxidase, washing of the slides with distilled water, coveting the slides with distilled water or a heavy metal solution, and brief microwave heating for several minutes. Following this procedure, slides are cooled, rinsed, and immunostained in a conventional fashion.

This method for restoring antigenicity is subject to certain limitations. First, it requires the use of a microwave oven to heat the tissue samples. Many laboratories may not be equipped with a microwave oven, and some tissue samples may not be suited to microwave heating. A need exists for an antigen retrieval method that can be used at room temperature, without any external heat source. In addition, the previously described procedure is especially suitable for tissues embedded in a hydrocarbon medium such as paraffin. It is not well suited for tissue sections embedded in celloidin, a preferred embedding medium for bony tissues. A need also exists for a method which is suitable for use with celloidin embedded tissues. Moreover, a need particularly exists for a method which may be used with decalcified bony tissue samples, since decalcified tissues are often refractory to the previously described method.

Methods and compositions are provided for restoring antigenicity for immunohistochmical analysis of aldehyde fixed tissue. The methods and compositions are especially useful with celloidin embedded tissue and with decalcified bony tissue. The method involves treating aldehyde fixed tissue with a chemical agent or agents that catalyze an essentially irreversible Mannich/Schiff's Base reaction which converts the aldehyde released thereby into a nonreactive form, i.e., into a non-aldehyde derivative. In this application such chemical agent or agents will be termed an "aldehyde releasing agent". In one aspect, the aldehyde releasing reagent comprises a nucleophilic reagent solution and the method involves. (1) treating aldehyde fixed tissue with the nucleophilic reagent solution, optionally containing a chaotropic agent, (2) removing excess nucleophile by neutralizing or rinsing the tissue sample, and (3) reacting the tissue with an immunoreactive reagent. In another aspect, the aldehyde releasing reagent comprises an oxidizing agent and the method involves (1) treating aldehyde fixed tissue with the oxidizing agent, (2) removing excess agent and (3) reacting the tissue with an immunoreactive reagent. In a third aspect, the aldehyde releasing reagent comprises an organic acid/base pair and the method in step (1) involves treatment with the organic acid/base pair. Exemplary organic acids are set forth below. Exemplary bases are sodium hydroxide and potassium hydroxide. Also provided is a kit for immunostaining of aldehyde fixed tissue. The kit minimally comprises a solution for restoring antigenicity comprising a solvent and an aldehyde releasing reagent. Optionally, the kit can also comprise a solution to remove excess aldehyde releasing reagent or a reagent immunostaining reagent.

The invention provides a method for restoring immunoreactivity of a tissue fixed with an aldehyde fixative agent and embedded in an embedding medium, the method comprising the initial step of contacting the tissue with an solution for restoring antigenicity comprising a solvent and an aldehyde releasing reagent. The aldehyde releasing reagent catalyzes reversal of the reaction between the aldehyde and biological components in the tissue, such as by catalyzing a reverse Mannich or a reverse Schiff base reaction. The released aldehyde reacts in a substantially irreversible manner to form a non-aldehyde derivative. Alternatively, the initial step can be broken down into two steps by adding the components of the antigenicity restoring solution separately: first adding solvent to remove at least part of the embedding medium, followed by adding an aldehyde releasing reagent, usually in the same or a different solvent. The initial step is followed by removing excess aldehyde releasing reagent from the tissue prior to immunostaining. Preferred embodiments of the invention provide a method for restoring immunoreactivity of a decalcified tissue fixed with an aldehyde fixative agent and embedded in an embedding medium comprising celloidin. Specific embodiments are described in complete detail below. A general theory of how the invention operates is set forth immediately below, along with a brief discussion of the physical and chemical processes that are believed to take place during the initial fixation process and the antigenicity restoring process of the invention. It must be understood that these are merely theories, and the invention can be fully practiced simply by reference to the descriptions of specific operations (and variations thereof) to be carried out by the practitioner.

The aldehyde fixation of tissue is believed to produce cross-linked proteins. This cross-linking is mediated by the reaction of aldehyde groups in the fixative with amino groups on amino acid residues of tissue proteins, such as lysine and the N-terminal a-amino acid group. The initial product of this interaction is an amino-aldehyde conjugate, either an imino Schiff base ($CHR_1{=}NR_2R_3$) or an aminomethylol ($CHR_1OHNR_2R_3$) intermediate. The intermediate may then undergo nucleophilic attack by susceptible neighboring amino acid groups, such as α-carbonyl methylene carbons having an acidic proton, nucleophilic heteroatoms, or electron rich aromatic rings. Prime nucleophiles include aromatic rings such as the ortho-position of the phenol ring of tyrosine, the C-2 position of the indole ring of tryptophan, and the imidazole ring of histidine; the a-carbons adjacent to

the side chain carboxylic acid groups of glutamate and aspartate; basic heteroatoms such as lysyl ε-amino groups; and neutral nitrogen atoms such as asparaginyl and glutaminyl amide groups and the indole ring nitrogen of tryptophan. Formally, all such reactions are types of or at least similar to Mannich reactions, at least inasmuch as the reactive electrophile is the intermediate amino-aldehyde conjugate species. These reactions result in a covalent bond between the electrophilic aldehyde carbon and a nucleophilic carbon or heteroatom.

The resulting cross-linking fixes proteins in a particular conformation and fixes the entire tissue by forming covalent bonds among adjacent proteins. The cross-linked proteins resist penetration by macromolecules such as antibodies. In addition, chemical modification of epitopes (which contain amine, amide, or aromatic amino acid residues) produces an altered structure unrecognizable to an antibody against that epitope.

The commonest aldehyde fixative is formaldehyde, which is unifunctional and produces cross-linking by direct contact between methylol-amino groups of lysine and adjacent susceptible amino acid target residues. However, other difunctional or polyfunctional cross-linking aldehydes are known. Of these the commonest is glutaraldehyde, a five carbon chain with aldehydes at both termini. This difunctional reagent provides additional opportunities for cross-linking, since the alkyl chain of the reagent functions as a spacer. The mechanism of reaction is believed similar, regardless of the particular aldehyde reagent used for fixation.

The method of the present invention provides a means for reversing or breaking at least some of these cross-linkages, thereby restoring the antigenicity of previously fixed proteins. The method involves treating the previously aldehyde-fixed tissue with a solution containing an aldehyde releasing reagent, which is believed to promote reversal of the Mannich-type reaction and other reactions between formaldehyde and tissue components. Antigenic restoration also may proceed as a result of limited proteolysis. In order to effectively restore antigenicity, the reagent need not reverse or break all aldehyde induced linkages. Partial breakage of cross-linkages loosens the fixed proteins sufficiently to permit penetration by antibodies. Particularly susceptible linkages are believed to be those produced between aminomethylol reactants and heteroatoms, such as amines and amides, or alpha-carbonyl methylene groups.

Most effective aldehyde releasing reagents for the practice of the invention are nucleophiles, preferably basic nucleophiles. An especially preferred nucleophile is hydroxide anion, which is conveniently supplied as an alkali metal hydroxide such as sodium or potassium hydroxide. Other convenient nucleophiles include primary, secondary, or tertiary amines, especially those with minimal steric hindrance to attack, such as piperidine or morpholine. Hydroxylamine and glycine are preferred. Other nucleophiles include thiols such as mercaptoethanol. Yet another nucleophile of interest is azide, e.g. sodium azide (NaN_3). In general, any nucleophile capable of promoting a reverse Mannich reaction will be capable of cleaving at last some protein cross-linkages, as such reagents will also catalyze reversal of other types of reactions caused by formaldehyde. The concentration of the nucleophile may vary widely, with more concentrated solutions acting more quickly. For short exposures, nucleophile concentrations of 0.5M or greater are usually preferred. In the case of NaOH in methanol, concentrations of one-tenth to one-half of saturation (approximately 0.6 to 3M) are preferred in most circumstances. For hydroxylamine, for example in the form of hydroxylamine hydrochloride, or for glycine, a 10% aqueous solution is preferred. Additional nucleophiles include hydrazine hydrate. And for hydrazine hydrate, an aqueous (v/v) solution in the range of about 2.0% to about 5.0% preferably 5.0%, is suitable.

Other effective aldehyde releasing reagents for the practice of the invention are oxidizing agents. Preferred oxidizing agents are hypochlorites and periodates, especially sodium hypochlorite or sodium periodate. Concentration of the oxidizing agent will vary with the reagent. For example, for sodium hypochlorite, an aqueous (v/v) solution in the range of about 0.01% to about 0.005%, preferably 0.005%, is suitable. For sodium periodate, an aqueous (v/v) solution in the range of about 0.1% to about 1.0%, preferably 0.1%, is suitable. Treating the sample with the oxidizing agent is believed to break the cross-linkages between the aldehyde and tissue components and converts the released aldehyde into a non-reactive form, for example by converting formaldehyde to formic acid.

We have found that certain acid/base pairs will function as an aldehyde releasing releasing agent within the confines of this invention. The following pairs are exemplary:

Organic Acid/Base Pair (Acid/Base)	Concentration (Acid/Base)	Minutes
Trichloroacetic Acid/NaOH	10% aqueous/10% NaOH in methanol	10/20
Toluene Sulfonic Acid/NaOH	5% aqueous/40% aqueous	30/30
Citric Acid/NaOH	10% aqueous/40% aqueous	30/30
Oxalic Acid/NaOH	20% aqueous/40% aqueous	30/30
Tartaric Acid/NaOH	10% aqueous/40% aqueous	30/30

The solvent for the aldehyde releasing reagent solution may be any solvent compatible with and capable of dissolving the aldehyde releasing reagent. Aqueous solutions are possible; and preferable where the aldehyde releasing reagent is an oxidizing agent or an organic acid/base pair. Organic solutions are preferable where the aldehyde releasing reagent is a nucleophile because they promote better penetration of the embedding medium. In addition, proteolytic fragments are insoluble in most organic solvents and therefore tend to remain in place on the slide.

A preferred solvent for use with celloidin embedded tissue sections is a polar organic solvent. Alcoholic solutions are preferred because they promote good penetration of the celloidin embedding medium and are good solvents for nucleophiles of interest, especially alkaline metal hydroxides. Lower alcohols are preferred, such is methanol, ethanol, propanol, and butanol; methanol is especially preferred. Polyols such as ethylene glycol and glycerol are also useful; they have the advantages of low volatility and greater viscosity, which permit them to remain on the slide for an extended period without evaporating or running off. Polar aprotic solvents such as dimethyl formamide (DMF) and dimethyl sulfoxide (DMSO) also may be used with appropriate nucleophiles. Furthermore, mixed solvent solutions are acceptable, provided the component solvents are compatible with the reagents and each other.

Two solvents may be used consecutively under some circumstances. For example, a celloidin embedded slide may be treated initially with methanol to solubilized the embedding medium, then treated with an aldehyde releasing reagent solution such as KOH in glycerol.

Nonpolar organic solvents are useful with tissues embedded in nonpolar media such as paraffin. In the case of a

nonpolar solvent such as toluene, a quaternary base is desirable since it is soluble in nonpolar solvents. Examples include tetraalkylammonium hydroxide (e.g. tetraethylammonium hydroxide) or a quaternary phosphonium salt. A nonpolar solvent and quaternary base may be combined with an immiscible polar aqueous phase in a phase-transfer reaction. The two phases may be applied to the tissue simultaneously, or the nonpolar phase may be applied first in order to facilitate solubilization of the paraffin embedding medium.

Additives may be included to enhance the desirable properties of the solution. Chaotropic agents, such as sodium thiocyanate, are preferred additives.

The embedded and fixed tissue sections are immersed in or covered by the aldehyde releasing reagent solution for periods ranging from several minutes to several hours. The optimal treatment period will vary depending concentration of aldehyde releasing reagent, type of solvent (if any), degree of penetration of the embedding medium by the solution, extent of tissue fixation, and temperature. For a particular combination of variables, an optimum time of treatment may be readily determined by treating tissue samples for different increments of time and measuring the extent of immunostaining. For a solution comprising methanolic sodium hydroxide at 25% saturation, a period of treatment of about 30 minutes is adequate for most tissue sections. Little improvement is seen for times less than about 5 minutes. At periods of contact longer than about 2 hours, the tissue may tend to detach from the slide; this is especially pronounced with 20 mm celloidin sections.

The treatment temperature affects reaction rate in the typical predictable pattern for chemical reactions, with elevated temperatures producing more rapid results. The method may be practiced conveniently at room temperature, and temperature control is not normally practiced.

After the treatment period with the aldehyde releasing reagent solution, excess reagent is removed from the tissue sample prior to immunostaining. This may be accomplished most conveniently by rinsing the tissue with solvent or solution which is free of the reagent. Multiple changes of rinsing solution are preferred. For greater preservation of tissue hydration and morphology characteristics, at least one of the rinsing solutions preferably will contain a mixture of the solvent used in the aldehyde releasing reagent solution and aqueous buffer. This facilitates re-equilibration of tissue with buffer. Preferably one or more washes with aqueous buffer will be performed prior to immunostaining. A preferred rinsing procedure uses at least one wash with buffer containing a detergent follows by one or more rinses with buffer containing a detergent followed by one or more rinses with buffer without detergent. The detergent may be any tissue compatible detergent, either ionic or non-ionic, although non-ionic detergents such as Triton X-100 are preferred.

As an alternative to the rinsing step, excess reagent may be neutralized with acid or a buffer. This alternative is most feasible when the solvent is similar or identical in composition to the solution to be used for the immunostaining. In general, immunostaining solutions are themselves aqueous buffers. For most applications, removal of excess base by rinsing will be preferable.

After removal of excess reagent, the tissue is immunostained by any conventional technique. A great variety of immunostaining procedures, reagents, and antibodies are known, many of which are commercially available. The procedures for restoring antigenicity described above leaves the tissue in a condition compatible with most immunostaining procedures. Typically, the tissue is incubated with a primary antibody against the antigen of interest, followed by treatment with a detectable label. The detectable label often includes a second antibody against the primary antibody; in turn, the second antibody may have the capacity to bind with a third species which is actually detected. These multiple levels of binding provide a means for amplifying the intensity of the detectable signal. In general, the antigen retrieval method described above does not interfere with the detection procedure.

The present method for restoring antigenicity using aldehyde releasing reagents may be used with essentially any embedding medium, including hydrocarbons, such as paraffin, and synthetic resins. However, it is particularly useful with celloidin, which is a traditional embedding medium for bony tissues. Celloidin is a pure form of pyroxylin, the low-nitrogen form of nitrocellulose. Celloidin is available from various commercial sources.

The described method preferably is performed with solution which either solubilizes or swells and softens the embedding medium. Most embedding media are supplied as solutions. Hence an appropriate solvent for the solution may be inferred based upon the solvation characteristics (e.g. hydrophobicity, polarity, hydrogen bond donor/acceptor potential) of the solvent supplied for the embedding medium. The solution need not employ a solvent identical to that used for the embedding medium. However, the identity of suitable embedding medium solvents provides a guideline to the appropriate characteristics for the aldehyde releasing solvent.

In the case of celloidin, the embedding medium is soluble in ether-alcohol mixtures, clove oil (comprising aromatic terpenes), alcohols, and acetone. Methanol is a suitable solvent, since it also solubilizes basic nucleophiles, and is a preferred solvent for use with celloidin embedded tissues.

Bony tissues in the past have presented particular problems, at least in part because of the decalcification treatment to which bone often is exposed during fixation. Conventional decalcification is performed with an acid such as trichloroacetic acid (Cl_3CCOOH). At present, immunohistochemical staining is not widely used in diagnostic and investigative pathology for routinely processed formaldehyde fixed, decalcified, and celloidin embedded bony tissues, as for example temporal bone sections. Several reported attempts to immunostain temporal bone sections focus on modified fixation, decalcification, and embedding protocols (Veldman et al., Advances in oto-immunology. New trends in functional pathology of the temporal bone, *Laryngoscop* (1987) 97:413; Huizing et al., Progress in temporal bone histopathology. I. Semithin 3–5 um sectioning of undecalcified human temporal bone after plastic embedding, *Acta Otolaryngol* (*Stockh*) (1985) Suppl 423:24; Veldman et al., Progress in temporal bone histopathology. II. Immuno-technology applied to the temporal bone, *Acta Otolaryngol* (*Stockh*) (1985) Suppl 423:29; Arnold, W., Immunohistochemical investigation of the human inner ear, *Acta Otolaryngol* (*Stockh*) (1988) 105:392; and Bauwens et al., Progress in temporal bone histopathology. III. An improved technique for immunohistochemical investigation of the adult human inner ear, *Acta Otolaryngol* (*Stockh*) (1990) Suppl 470:34).

The disclosed method simultaneously neutralizes any residual acidity from decalcification, which might impede restoration of antigenicity using water or other solvent by itself with bony specimens. As the accompanying experi-

mental results demonstrate, the disclosed method permits effective immunostaining of temporal bone sections which have been routinely processed and embedded. This is significant because human temporal bone collections amounting to 8,000 and 13,000 specimens exist in Europe and the United States, respectively (Schuknecht, *Ann. Otol. Rhinol. Laryngol*, (1987) 96 (Suppl. 130):1). These collections provide an excellent research base for understanding otopathology by light microscopy.

A kit for use in immunostaining of a tissue can be provided to simplify practice of the method described above. The kit will minimally contain a receptacle adapted to hold one or more individual reagent containers and at least a first container containing (1) an aldehyde releasing reagent solution comprising a solvent and an aldehyde releasing reagent or (2) the aldehyde releasing reagent in an amount appropriate to make up the desired concentration when solvent from another container is used to fill the aldehyde releasing reagent container to a predetermined level. In most cases, the kit will also contain a second container containing (1) an immunostaining reagent or (2) a wash solution for removing excess aldehyde releasing reagent solution, or containers with both such materials. Wash solutions are typically buffered solutions that do not further dissolve or swell the embedding medium, such as aqueous buffered solutions. The solvent in the wash solution will be capable of dissolving the solvent and aldehyde releasing reagent used in the antigen retrieval solution. The immunostaining reagent generally comprises an antibody and staining moiety. Such reagents are well known in the art and require no further description here. Specific examples are given in the general examples of the invention set out below. Appropriate instructions for carrying out the method of the invention will also be included in the kit.

The invention now being generally described, the same will be better understood by reference to the following detailed examples which are provided for illustration and are not to be considered as limiting the invention unless so specified.

EXAMPLE 1

A Nucleophile as an Aldehyde Releasing Reagent

1. Materials and Methods

A total of 60 celloidin-embedded human temporal bone sections were obtained from the Eastern National Temporal Bone Bank at Massachusetts Eye and Ear Infirmary (Table 1). Most sections were processed routinely by either Heidenhain-Susa or 100% formalin fixation and decalcified by 5% trichloroacetic acid as described previously (Schuknecht HF. Pathology of the Ear. Cambridge, Mass.: Harvard University Press. 1974). Only one case was processed by a modified method using 10% neutral buffered formalin fixative and EDTA decalcification.

The monoclonal antibodies used are listed in Table 2. All antibodies were obtained from BioGenex Laboratories (San Ramon, Calif.). Most slides were stained with Super Sensitive biotin-streptavidin kits (SSBSA) from BioGenex. A few slides were stated by ABC kits purchased from Vector Laboratories, Inc. (Burlingame, Calif.).

2. Preparation of Aldehyde Releasing Reagent Solution

Sodium hydroxide (NaOH) in methanol solution provides one formulation of the basic reagent used in these experiments. NaOH, 50–100 grams, was added to 500 ml methanol in a brown colored bottle. The solution was mixed by shaking and stored at room temperature for 1–2 weeks to settle. The upper layer of liquid was removed carefully from the precipitate, and diluted 1:3 in methanol. Prior to use, 0.01% sodium thiocyanate optionally was added.

3. Treatment of Tissue Sections

The celloidin-embedded temporal bone sections were washed in distilled water for 10 minutes, mounted on 0.1% poly-L-lysine (Sigma) coated slides. The slides were immersed in one-quarter saturated NaOH-methanol solution (saturated methanolic NaOH, approximately 6M, diluted 1:3 with methanol) alone or with added 0.01% sodium thiocyanate, for 30 minutes. Slides were rinsed for 15 minutes in two changes of 100% and 70% methanol and two changes of phosphate buffer saline (PBS), followed by treatment with 0.3% Triton X-100 for 10 minutes and rinsing in PBS again.

4. Immunostaining Procedures

Treatment was followed by a three-step immunostaining technique using either the SSBSA or the ABC method, as previously described (Shi et al., *J. Histochem. Cytochem.* (1991) 39:741). Briefly, slides were incubated with primary antibody overnight at room temperature followed by a 40 to 60 minute incubation with link (BioGenex Super Sensitive biotinylated anti-mouse immunoglobulin or Vector biotinylated anti-mouse immunoglobulin). Label (BioGenex Super Sensitive Alkaline phosphatase conjugated streptavidin or peroxidase conjugated streptavidin and Vector ABC) was added for 40 to 60 minutes. Slides were rinsed between incubations in three changes of PBS for 15 minutes. Either fast red or DAB chromogen was used as substrate. The immunostaining results were controlled by light microscopy.

The primary antibody was replaced with either nonspecific mouse ascites or PBS for negative control slides.

TABLE 1

Routine celloidin-embedded human temporal bone sections used (N = 60)

Code	No. of sections	Fixation	Decalcification	Cut	Stored
P.F.	10 (L&R)	NBF	EDTA	Recent	<1 yr
C.B.	10(R)	H-S	TCA	1989	<2 yrs
R.L.	10(L)	F	EDTA	1989	<2 yrs
G.H.	10(R)	H-S	TCA	1986	4 yrs
J.P.	10(R)	H-S	TCA	1982	8 yrs
R.Y.	10(L)	F	TCA	1960	30 yrs

a. L = left side. R = right side of temporal bone.
b. NBF = 10% neutral buffered formalin. H-S = Heidenhain-Susa fixative. F = 10% formalin.
c. TCA = trichloroacetic acid.

5. Results

The immunoreactivity of 15 monoclonal antibodies used on routinely processed celloidin-embedded sections is summarized in Table 2. The staining results showed strong positive staining for 7 monoclonal antibodies, moderate positive staining for four antibodies, and weak positive staining for one antibody. Three antibodies showed negative results. There was no significant difference in immunoreacfivity between various sections. All negative control slides (PBS or nonspecific mouse ascites) showed negative staining. The intensity of immunostaining obtained by the SSBSA system was stronger than that obtained by the ABC system.

The immunoreactivity of monoclonal antibody to kernfin (AEI and NCL-5D3), vimentin, neurofilament, muscle specific actin, S-100 protein, neuron specific enolase (NSE), glial fibrillazy acidic protein (GFAP) and others showed

strong positive results. In a routine celloidin embedded human temporal bone section that was immunostained with monoclonal anti-keratin antibodies NCL-5D3 and AE1 following treatment with aldehyde releasing reagent, all epithelial cells lining the coacher duct were distinctly labeled by the anti-keratin antibodies. Keratin localization within the organ of Corti was also discernible. The keratin immunoreactivity showed so distinctively that all epithelial cells were precisely demonstrated by the immunohistochemical staining.

Other celloidin embedded temporal bone sections immunostained with monoclonal antibodies after treatment with aldehyde releasing reagent solution also showed strong positive results. Antibody against neuron specific enolase (NSE) was localized in spiral ganglion neurons and neurofibers. Anti-NSE immunostaining was localized within inner hair cells in the organ of Corti but was absent in outer hair cells except at the bottom of the outer hair cells where it occurred possibly in synapses and terminal nerve branches. Glial fibrillary acidic protein (GFAP) was localized along the glial-Schwann junction to the brain side only. Desmin was localized in tensor tympanic muscle by anti-desmin antibody. This labelling with anti-desmin antibody was not possible using micro-dissection methods (Bauwens et al., *Acta Otolaryngol.* (1990) Suppl. 470:34). The skin and appendages of dermis in the external auditory canal were also stained positively by some antibodies such as keratin and actin. Tubulin was widely localized in most epithelial and mesenchymal cells of whole temporal bone.

TABLE 2

Immunohistochemical Staining on Routine Processed, Celloidin-Embedded Human Temporal Bone Sections

Monoclonal Antibody	Results
Keratin:	
AEI	+++
NCL-5D3	+++
Vimentin	+++
NF	+++
GFAP	+++
Desmin	++
Myoglobin	++
a-Tubulin	++
b-Tubulin	+
Muscle Specific Actin	+++
Chromogranin	–
a-Actinin	–
EMA	–
NSE	++
S-100	+++

Immunoreactivity was scored on a scale of – to +++, – being non-reactive and +++ being highly reactive.

EXAMPLE 2

An Oxidizing Agent as an Aldehyde Releasing Reagent

1. Preparation of Solution

Sodium hypochlorite (NaClO) in distilled water solution provides one formulation of the basic reagent used in these experiments. NaClO, 0.01–0.005 grams, was added to 100 ml of distilled water in a brown colored bottle. The solution was mixed by shaking and storing at room temperature.

2. Treatment of Tissue Sections

The deparaffinized tissue slides were washed in distilled water for 10 minutes. The slides were immersed in an aldehyde releasing reagent solution, i.e., dilute sodium perchlorate (aqueous) solution for 10 minutes.

3. Immunostaining Procedures and Results

Treatment was followed by a three-step immunostaining technique. Briefly, slides were incubated with primary antibody overnight at room temperature followed by a 20–30 minute incubation with a link (BioGenex Super Sensitive biotinylated anti-mouse immunoglobulin or Vector biofinylated anti-mouse immunoglobulin). A label (BioGenex Super Sensitive Alkaline phosphatase conjugated streptavidin or peroxidase conjugated streptavidin and Vector ABC) was added for 20–30 minutes. Between incubations, slides were rinsed in three changes of PBS for 15 minutes. Either fast red or DAB chromogen was used as a substrate. The immunostaining results were controlled by light microscopy. Positive results were seen.

EXAMPLE 3

An Organic Acid/Base Pair as an Aldehyde Releasing Reagent

The deparaffinized tissue slides were washed in distilled water for 10 minutes. The slides were loaded with a 5–10% aqueous solution of a citric acid. After waiting for 10–30 minutes, the slides were loaded with a 10% methanolic/aqueous solution of sodium hydroxide (prepared as set forth in Example 1) for 10–30 minutes. Immunostaining proceeded as set forth in Example 2 and positive results were seen.

The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

We claim:

1. A method for restoring immunoreactivity of a tissue fixed with an aldehyde fixing agent and embedded in an embedding medium, said method comprising the steps of:

a) contacting said tissue with a solvent for said embedding medium and an aldehyde releasing reagent, which reagent releases aldehyde from said tissue by reacting said aldehyde in a substantially irreversible manner to form a non-aldehyde derivative; and

b) removing or neutralizing excess aldehyde releasing reagent from said tissue.

2. The method of claim 1, wherein said solvent is selected from the group consisting of methanol, ethanol, propanol, butanol, ethylene glycol, and glycerol.

3. The method of claim 1, wherein said aldehyde releasing reagent is selected from the group consisting of nucleophilic bases, oxidizing agents and organic acid/base pairs.

4. The method of claim 1, wherein said aldehyde releasing reagent is an oxidizing agent.

5. The method of claim 1, wherein said aldehyde releasing reagent is a nucleophilic base.

6. The method of claim 1, additionally comprising in step a) contacting said tissue with a chaotropic agent.

7. The method of claim 1, wherein said tissue comprises decalcified tissue.

8. A kit for use in immunostaining of a tissue fixed with an aldehyde fixing agent and embedded in an embedding medium, said kit comprising:

a) a first container containing an aldehyde releasing reagent solution comprising an aldehyde releasing reagent selected from the group consisting of nucleophilic bases, oxidizing agents and organic acid/base pairs and a solvent for the aldehyde releasing reagent which is also capable of solubilizing the embedding medium; and

b) a second container containing (1) an immunostaining reagent or (2) a wash solution for removing excess aldehyde releasing reagent.

9. The method of claim **3** wherein said aldehyde releasing reagent is an aqueous or organic solution.

10. A kit for use in immunostaining of a tissue fixed with an aldehyde fixing agent and embedded in an embedding medium, said kit comprising:

a) a first container containing an aldehyde releasing reagent selected from the group consisting of nucleophilic bases, oxidizing agents and organic acid/base pairs;

b) a second container containing a solvent for solubilizing the embedding medium; and

c) a third container containing (1) an immunostaining reagent or (2) a wash solution for removing excess aldehyde releasing reagent.

11. The kit of claim **8** or **10** wherein said aldehyde releasing reagent comprises a nucleophilic base.

12. The kit of claim **8** or **10** wherein said aldehyde releasing reagent comprises an oxidizing agent.

13. The kit of claim **8** or **10** wherein said aldehyde releasing reagent comprises an organic acid/base pair.

14. The kit of claim **8** or **10** wherein said solvent is selected from the group consisting of methanol, ethanol, propanol, butanol, ethylene glycol, and glycerol.

15. The kit of claim **8** or **10** wherein said second container contains an immunostaining reagent comprising an antibody.

* * * * *

EXHIBIT B

US005244787A

United States Patent [19]

Key et al.

[11] Patent Number: 5,244,787

[45] Date of Patent: Sep. 14, 1993

[54] **ANTIGEN RETRIEVAL IN FORMAL IN FIXED TISSUES USING MICROWAVE ENERGY**

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[21] Appl. No.: 649,036

[22] Filed: Jan. 31, 1991

[51] Int. Cl.[5] C12Q 1/00; G01N 33/535

[52] U.S. Cl. 435/7.9; 424/3; 435/7.94; 435/7.92; 436/518; 436/519

[58] Field of Search 424/3; 435/7.9, 7.94; 436/518

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[57] **ABSTRACT**

A method of immunologically staining a formalin-fixed tissue preparation, which comprises (a) subjecting a formalin-fixed tissue preparation to microwave energy while the tissue preparation is submersed in water for a time sufficient to increase immunostaining efficiency; (b) removing the tissue preparation from the water and cooling; and (c) contacting the tissue preparation with an immunological staining reagent.

18 Claims, No Drawings

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ANTIGEN RETRIEVAL IN FORMAL IN FIXED TISSUES USING MICROWAVE ENERGY

TECHNICAL FIELD

This application is directed to the field of tissue staining and is particularly directed to antibody-directed staining of formalin-fixed tissue

BACKGROUND

Growing interest in immunohistochemical staining procedures has led to the development of a wide range of highly specific immunostains which are of value to the surgical pathologist in diagnostic and investigative studies (1, 2). Although formalin remains the most popular fixative used in pathology, it is clear that this fixative is not always the best choice for preserving antigenicity of tissues to be used in immunohistochemical procedures Despite numerous studies on the intermolecular crosslinks formed between formalin and proteins (3, 4), the molecular mechanism underlying tissue fixation is not well understood (5).

The demand for a broader selection of antibodies which can be used for immunohistochemical staining on routine formalin-fixed, paraffin-embedded tissues has stimulated efforts to develop antibodies which can recognize formalin-resistant epitopes Although this strategy has been effective in developing many useful antibodies, it has not been entirely satisfactory in resolving all problems. A persistent concern in immunopathology is choosing the correct fixative and duration of fixation that will provide the maximum preservation of tissue morphology with minimum loss of antigenicity.

One approach to resolve this dilemma was the introduction of protease digestion of formalin-fixed sections to unmask antigenic sites hidden by cross-linked proteins (6, 7). However, Leong, et al , (8) showed that, aside from cytokeratins and desmin, digestion with trypsin did not improve immunostaining of the other antigens studied. Presently it is not clear whether or not the formalin-induced cross-linking of proteins is a reversible chemical reaction. However, a recent study concerning formalin sensitivity of a GFAP epitope supported the hypothesis that the sensitivity of some epitopes was not due to the direct effect of the aldehyde, but rather due to the binding of other molecular structures to the epitope (9).

Clearly the ability to bind immunostaining reagents with epitopes masked by formalin fixation (referred to here as antigen-retrieval) could significantly expand the range of antibodies useful in immunohistochemistry as well as reduce the incidence of false negative staining in over-fixed tissues. Additionally, antigen retrieval could provide greater diagnostic accuracy by improving immunohistochemical procedures. With these goals in mind we studied the effects of microwave oven heating of tissue sections in the presence and absence of metal solutions. The dramatic enhancing effect of this treatment on antigen recovery and immunohistochemical staining was particularly surprising considering the deleterious effects that high temperatures are presumed to have on protein antigens.

The microwave oven has previously been used for tissue fixation (8, 10, 11) and for rapid histochemical and immunohistochemical staining (12–19). One recent report has also observed enhanced immunohistochemical staining following microwave drying of slides (20). However, in these cases, only short periods of irradiation and low temperatures were used To the best of our knowledge, no one has previously accomplished antigen retrieval by the use of the microwave oven alone or with metal solutions. Similarly, no studies have indicated that immunohistochemical staining intensity could be increased by heating slides in water with microwaves to temperatures of approximately 100° C.

The use of heavy metal salts in combination with formalin for tissue fixation has recently been introduced (21, 22). Some studies have demonstrated the superiority of zinc formalin as a fixative for antigen preservation (22) Furthermore, when routine formalinfixed tissues were re-fixed in zinc formalin, immunoreactivity was improved (23).

Although little has been published on the molecular changes in amino acids and other compounds that occur after microwave treatment (24), Stroop, et al., (25) demonstrated that microwave treatment of radiolabeled DNA probes allowed these probes to be diluted about 20 times more than when these probes were denatured by conventional heat.

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SUMMARY OF THE INVENTION

It is an object of the present invention to provide an improved technique for immunological staining of formalin-fixed tissue.

It is particularly desirable to provide a technique that is generally applicable and which is simple to carry out with readily available equipment.

Accordingly, these and other objects of the invention as will hereinafter become more readily apparent by reference to the following detailed description of the invention have been accomplished by providing a method of immunologically staining a formalin-fixed tissue preparation, which comprises (a) heating a formalin-fixed tissue preparation using microwave energy while the tissue preparation is submersed in water, (b) removing the tissue preparation from the water and cooling, and (c) contacting the tissue preparation with an immunological staining reagent or series of reagents.

DESCRIPTION OF SPECIFIC EMBODIMENTS

The present invention provides a new approach for retrieval of antigens from formalin-fixed, paraffin-embedded tissues and their subsequent staining by immunohistochemical techniques. This method of antigen retrieval is based on microwave heating of tissue sections. The tissue sections are immersed in water and are preferably attached to microscope slides. Only moderate heating is required to improve immunostaining, but heating to temperatures sufficient to boil a solution of water, or plain water, in which the tissue preparation is submersed are preferred. This temperature is preferably at about 100° C. (±5° C.) and preferably takes place in the presence of metal ions.

Among 52 monoclonal and polyclonal antibodies tested by the method of the invention, 39 antibodies demonstrated a significant increase in immunostaining, nine antibodies showed no change, and four antibodies showed reduced immunostaining. In particular, excellent immunostaining results were obtained with monoclonal antibody to vimentin as well as several different keratin antibodies on routine formalin-fixed tissue sections after pre-treatment of the slides with this method. These results showed that antigen retrieval process of the invention has a number of useful properties: 1) enzyme pre-digestion of tissues is not necessary, 2) incubation times with primary antibodies can be significantly reduced and dilutions of primary antibodies can be increased, 3) adequate staining can be achieved in tissues fixed for from, e.g., two weeks up to two years in formalin which fail to stain by conventional methods, and 4) certain antibodies, which are typically unreactive with formalin-fixed tissues, give excellent staining using the techniques of the invention.

Although the method of the invention does not increase immunostaining for every antibody-antigen pair, as indicated by the statistics above the technique is none the less generally useful as it increases immunostaining in about ¾ of antibody-antigen pairs tested so far. Similar results are expected for other antibody-antigen pairs. Furthermore, whether immunostaining will be increased for any particular staining antibody can readily be determined on a small scale before being applied generally for use with a particular antibody. In other words, for each new antibody to be used in a staining process, the method of the invention is carried out and compared to immunostaining in the absence of microwave heating Detailed examples of how this process is carried out are set forth below. If immunostaining is increased, the process of the invention will be effective generally for that antibody-antigen pair in the future. The testing is straight forward and easily accomplished in about two hours, during which time several different antibodies can be tested concurrently.

Although the mechanism by which microwave oven recovery of antigens takes place is not clear (since this treatment did not affect alcohol-fixed paraffin sections), it is possible that the crosslinking of proteins caused by formaldehyde is altered by microwave heating. The morphology of tissues after microwave oven irradiation for 10 minutes showed no significant changes based on light microscopic analysis. However, some ultrastructural alterations, as suggested by a recent study (26), may be present resulting from fixing (rather than antigen recovery).

When compared to protease digestion, the influence of microwave pre-treatment was clearly superior, par-

ticularly in long-term formalin-fixed tissues. Particularly significant was our finding that tissues fixed in formalin for two weeks up to two years could still be immunostained following antigen retrieval even though enzyme pre-digestion failed to restore immunoreactivity.

A comparison between microwave oven treatment and conventional heating methods (conventional oven) showed significantly different immunostaining results. While pre-treating the slides to boiling (100°±5° C.) by microwave oven resulted in excellent immunostaining, poor results were obtained by pre-treating the slides with boiling water using conventional heat. It may be that some additional physical effects from microwave irradiation are important factors in achieving enhancement.

Furthermore, we found that the use of metal solutions in combination with microwave oven heating could substantially improve the immunoreactivity above that achievable with no treatment or microwave treatment in distilled water. This was particularly evident in tissues which had been fixed in formalin for greater than 24 hours. Some antibodies used in these studies could be diluted hundreds of times further than their usual concentrations. In addition, these antibodies could be used at their usual concentrations, but the incubation times of the antibody reaction on the tissues could be shortened by approximately 6-fold with no loss in sensitivity.

Different metal ions can be used in this preferred embodiment of the invention. Divalent metal ions are preferred, particularly divalent transition metals and group 4a metals, particularly tin and lead. Two particular preferred metal ions are zinc and lead. Lead ions are more effective than zinc during microwave oven treatment, since the lead solution demonstrated stronger immunoreactivity with less background.

The practice of the present invention is quite straightforward and requires no special equipment or formulations and no modification of previously used immunological staining techniques. The process is carried out on a formalin-fixed tissue preparation prepared by any fixing process that uses formalin (or a different formaldehyde derivative or form) as a tissue-fixing agent. Since the tissue preparation that is used in the present invention is prepared prior to the process of the invention, the preparation of such formalin-fixed, paraffin-embedded tissue samples is not part of the present invention and, in fact, is well known to those of ordinary skill in the art. An example is described below for purposes of illustration.

Additionally, certain steps can occur after fixing (and storage, if storage takes place) of the tissue preparation but prior to the microwave-heating process. Typically, paraffin is removed from the paraffin-embedded tissue, for example by melting of the paraffin (which has a melting point of approximately 55° C.–60° C. depending upon the type of paraffin) or dissolving the paraffin in an appropriate solvent, such as chloroform or xylene. If an enzyme label, such as horseradish peroxidase, is used as a label in the immunostaining process, background enzymatic activity can be reduced; for example, endogenous peroxidase can be blocked with a solution of hydrogen peroxide, after which excess hydrogen peroxide is washed from the tissue preparation. The tissue preparation, which is typically on a glass slide, is then placed in water or an appropriate aqueous solution for the microwave heating process.

Since the process of the present invention can take place either in water or in an aqueous solution (such as a buffered solution or a solution containing metal ions, as discussed below), this step of the process is generally referred to in this specification as placing the tissue preparation into "water" rather than the more cumbersome "water or an aqueous solution " Reference solely to "water" is therefore understood in this specification to indicate either water or an aqueous solution. If an aqueous solution is used, the solution will preferably be at least 90% H_2O by weight, more preferably at least 95% H_2O, and most preferably at least 98% H_2O.

Although improved antigen recovery (relative to no treatment) is achieved by microwave heating in water, it is preferable to carry out the microwave heating step in an aqueous solution containing a heavy metal ion, particularly a divalent metal ion. Zinc and lead ions are particularly preferred, with lead ions generally producing the best results. The counter ion used is immaterial as long as a counter ion is selected that will provide solubility in water. Typical metal-ion solutions contain about 0.5–2% by weight of the salt. Concentrations of about 1% by weight are preferred.

The heating process is preferably carried out in a loosely covered container, as steam will sometimes be generated during the heating process and must be allowed to escape. On the other hand, completely open containers are not preferred, since a loose-fitting cover helps retain water during the heating process. A jar with a loose-fitting screw cap (or any container with a similar degree of "openness") is a preferred container in which to submerse the tissue preparation in the water for treatment.

A typical commercially available microwave oven can be used for the heating step. Alternatively, special equipment can be designed specifically for the process of the present invention. The microwave power setting will vary with the specific design of the equipment and the amount of material being treated. Microwaves are (by definition) in the frequency range of from 1 to 50 GHz. All commercially available microwave presently produce microwaves with a frequency of 2.45 GHz. Power levels are selected so as to provide between 300 to 800 W of power, preferably 600–750 W. At the optimal setting of 720 W, the temperature of 50 ml of water will increase at a rate of 1.67° C./second. Heating can be continuous or interrupted. For example, a 10-minute heating time can be divided into two 5-minute cycles with an interval of 1 minute between cycles in order to check on the fluid level in the containers during the heating process.

The total time that heating takes place can vary significantly. Even a relatively small amount of heating (a few seconds) improves antigen recovery in most cases. However, heating times of at least 1 minute are typical, preferably at least 5 minutes, more preferably about 10 minutes, usually less than 20 minutes, more generally less than 15 minutes.

The temperature at which heating takes place is generally that of the boiling point of the water or aqueous solution in which the heating takes place, although heating without reaching the boiling point is moderately effective. Since water boils at 100° C. (at 1 atmosphere of pressure), this is typically the temperature at which heating takes place in distilled water. With aqueous solutions, the boiling point is slightly elevated and depends on the concentration of the solution (as can be determined by Raoult'law). Solutions are generally

sufficiently dilute so as not to raise the boiling point to more than 105° C.

After heating, containers with the immersed tissue preparation are removed from the microwave source and allowed to cool, e.g., for 15 min. The temperature to which the materials cool is not particularly important, except for ease of handling. However, the cooling process is useful as additional antigen retrieval continues during this time. After sufficient cooling to allow handling, the tissue preparations are usually rinsed with distilled water to remove any excess aqueous solution or metal salts from their surface. If they are heated in distilled water, no such rinsing is required However, a final rinsing in a buffer prior to immunological staining is preferred in order to provide physiological conditions suitable for antigen-antibody bonding on the surfaces of the tissue preparation. Buffers particularly provide a pH of 6.5 to 8.5, or preferably about 6.8 to 8.0, and most preferably about 7.0 to 7.6. Numerous physiological buffers are commercially available through biological supply houses. Specific buffers may be selected according to the antibody being used. Since such selection of buffers relates to the immunostaining process rather than to the antigen recovering process of the present invention, buffer selection is not considered to be a part of the present invention.

The immunological staining process is no different from that previously known. No modification of techniques is required for using an immunological staining process with the steps of the present invention described herein.

The invention now being generally described, the same will be better understood by reference to the following detailed examples, which are provided for purposes of illustration only and are not to be considered limiting of the invention unless so specified.

EXAMPLES

Preparation of Tissues and Paraffin Blocks

Fresh tissues were obtained from the Cooperative Human Tissue Network (CHTN, Columbus, Ohio) and were fixed immediately in either 10% neutral buffered formalin or 90% ethanol Fixed tissues were dehydrated in ethanol, cleared in xylene, and embedded into paraffin blocks. Some tissues, which had been fixed in formalin for from two weeks up to two years, were obtained from the Department of Pathology, National Medical Center of the City of Hope (Duarte, Calif.) and Hartford Hospital (Hartford, Conn.). Five-micron sections were cut and mounted on poly-L-lysine-coated slides. Poly-L-Lysine is a positively charged, high molecular weight polymer of the amino acid lysine which, when coated onto microscope slides, acts as a tissue adhesive bonding the tissue to the slide. The use of a tissue adhesive is a useful modification of this procedure as tissues are prone to detach from their slides upon exposure to high heat in aqueous solutions. Other tissue adhesive that could withstand microwave heating of slides are acceptable.

Sources of Reagents

All polyclonal and monoclonal antibodies listed in Table 1 were obtained from BioGenex Laboratories (San Ramon, Calif.). Unless otherwise specified the detection system for the immunohisto-chemical staining was the Super Sensitive system, also from BioGenex. In some experiments a MultLink Detection System (Biogenex) was also evaluated. Both horseradish peroxidase with AEC and alkaline phosphatase with Fast Red were used.

Standard Heating Step Using Microwave Oven

A Toshiba model ER-855BT operating at a frequency of 2.5 GHz with nine power level settings was used at the highest power setting (720 watts). Three Coplin jars were filled (50 ml each) with saturated lead thiocyanate, placed in the center of the microwave oven, and heated for two five-minute cycles with an interval of one minute between cycles. Because the number of Coplin jars placed in the microwave oven influences the temperature, three jars were always used and were always placed in the same positions in the following examples. However, in other tests using only one or two jars the process of antigen retrieval was similar to the results seen with three jars, even though the temperature increase was more rapid.

Standard Protocol for Antigen Retrieval

Although different antigens may behave differently under similar conditions of treatment, the following protocol was found to be acceptable for most antigens tested in this study. The steps for antigen retrieval were as follows:

1. Tissue sections were deparaffinized and rehydrated.
2. Endogenous peroxidase was blocked with 3% H_2O_2 for five minutes.
3. Slides were washed with distilled water for five minutes.
4. Slides were then placed in a plastic Coplin jar containing either distilled water, a metal solution of saturated lead thiocyanate, or 1% zinc sulfate.
5. Jars were covered with a loose fitting screw cap and heated in the microwave oven for either five or 10 minutes. Sometimes a 10-minute heating time was divided into two five-minute cycles with an interval of one minute between cycles in order to check on the fluid level in the jars.
6. After heating, the Coplin jars were removed from the oven and allowed to cool for 15 minutes.
7. Slides were then rinsed in distilled water twice and in PBS for five minutes.
8. Treated slides were immunostained as described below.

Immunohistochemistry

Immunohistochemical staining was performed according to the manufacturer's instructions. Briefly, all incubations were performed at room temperature as follows: 1) primary antibodies were incubated from 30 minutes to 24 hours according to the manufacturer's instructions, 2) link antibody was incubated for 20 minutes, 3) streptavidin-conjugated enzyme was incubated for 20 minutes, 4) peroxidase substrate was incubated for 5 minutes, or alkaline phophatase substrate was incubated for 20 minutes.

Enzyme Digestion

In some cases deparaffinized tissues were pretreated by protease digestion prior to application of the primary antibody. Tissue sections were incubated with 0.1% trypsin in phosphate buffer saline (PBS) for 30 minutes at 37° C. Following enzyme digestions, slides were rinsed in PBS and immunostained as previously described.

Nonimmune rabbit serum or nonspecific mouse ascites were used as negative controls for rabbit and mouse primary antibodies, respectively. Contribution of nonspecific staining of primary antibody was evaluated by substitution of the primary antibody with the negative controls or with PBS.

For comparison, a conventional oven was also used to heat the slides. The solutions were heated to the specified temperature and then the slides were placed in the preheated solutions for five to 10 minutes and then treated as previously described for the microwave procedure.

Results of Antigen Retrieval and Staining Processes

Immunostaining results using 52 different primary antibodies on tissues treated for antigen retrieval are summarized in Table 1. Most antibodies tested showed increased intensity of immunostaining following microwave oven heating in the presence of either distilled water or metal solutions. In general, the intensity of immunostaining was stronger with the metal solutions, particularly using the lead solution. Some cases, such as with monoclonal antibody to IgD, the use of zinc sulfate solution caused strong background staining of tonsil epithelium and some false positive staining of lymphocyte nuclei. This type of false positive staining was not observed with the lead solution.

TABLE 1

Immunostaining of Formalin-Fixed, Paraffin-Embedded Tissue Following Antigen Retrieval

Improved Staining	No Change	Decreased Staining
Pan-cytokeratin (F12-19)	Tubulin (P)	Ferritin (P)
Cytokeratin (AE1)	Desmin (P)	Ferritin (M3.170)
Cytokeratin (AE3)	Desmin (33)	C3 (P)
Cytokeratin (AE8)	Myoglobin (P)	Gastrin (P)
Cytokeratin 7 (CK7)	Myoglobin (MG-1)	
Cytokertain 8,18,19 (5D3)	β-Endorphin (P)	
IgD (IADB6)	α-1-Antitrypsin (P)	
GFAP (P)	Transferrin (HT1/13.6.3)	
GFAP (GA-5)	Calcitonin (P)	
NF (2F11)		
CEA (P)		
CEA (SP-651)		
VIP (P)		
Serotonin (P)		
Estrogen receptor related protein (D5)		
C-erb-B2 (CB11)		
CMV (P)		
Albumin (P)		
Macrophage (LN5)		
Blood group A (81 FR2.2)		
Blood group B (81/11)		
Cathepsin B (P)		
Vimentin (V9)		
NSE (P)		
NSE (MIG-N3)		
Chromogranin (LK3H10)		
ACTH (R)		
α-hCG (02-310-94)		
PSA (8)		
Thyroglobulin (P)		
Factor VIII (P)		
Myeloid, CD15 (Tü9)		
T-cell (MT1)		
T-cell (MT2)		
B-cell (MB1)		
B-cell (MB2)		
Kappa chain (KP-53)		
Lambda chain (HP6054)		
AFP (A-013-01)		

TABLE 1-continued

Immunostaining of Formalin-Fixed, Paraffin-Embedded Tissue Following Antigen Retrieval

Improved Staining	No Change	Decreased Staining
Total: 39	9	4

NOTE:
(P) = Polyclonal antibodies; others are monoclonal

Best results were obtained when slides were heated in the microwave oven using the intermittent heating method of two five minute cycles with an interval of one minute between the heating cycles. Another advantage of this method was that additional solution could be added to the jars if necessary.

Heating slides in distilled water or metal solutions by conventional heat in an oven also resulted in some increased immunostaining; however, there were noticeable differences (Table 2).

For tissues fixed in formalin for 24 hours or longer, heating the slides by microwave oven with or without metal solutions was clearly better than conventional heat with metal solutions although some enhanced staining was seen in the latter case. The intensity of positive immunostaining obtained by using conventional heat was consistently weaker than that obtained by microwave oven heat.

Sensitivity of Antigen Retrieval Method

In order to demonstrate the increased sensitivity achievable with this method, selected antibodies were tested at titers which failed to produce positive stains when tested by a conventional immunostaining procedure. Furthermore, immunoreactivity could not be demonstrated with these antibodies even with the use of trypsin pre-digestion of tissues. When these antibodies were then tested on the same tissues following antigen retrieval, strong immunostaining was observed (Table 3).

Specificity of the Angiten Retrivel Method

The specificity of the antigen retrieval method was tested by immunostaining tissues known to either contain or lack certain antigens. For these studies tissues were immunostained with monoclonal antibodies to cytokeratin 7 or estrogen-receptor related protein p29. Both of these antibodies detected formalin sensitive but ethanol resistant epitopes, Tissues were first categorized as being antigen positive or antigen negative by immunostaining frozen sections of each tissue fixed in ethanol. The remainder of the tissues were then fixed in formalin and embedded into paraffin. When paraffin-embedded tissues known to contain antigen were tested with antibody to cytokeratin 7, no staining occurred in any formalin-fixed tissue regardless of the length of time of fixation. Similarly with antibody to p29, no staining occurred in antigen-positive tissues which had been fixed in formalin for 48 hours or longer. Although neither antibody detected antigen in formalin fixed tissues prior to antigen retrieval, after retrieval both gave strong staining of their respective antigens. Furthermore, when formalin-fixed, paraffin-embedded tissues which were negative for these antigens were immunostained for cytokeratin 7 or p29, no staining occurred either with or without antigen retrieval.

Antigen Retrieval in Long-Term Formalin Fixed Tissues

Thirty-nine different tissues which had been fixed in formalin for periods of time ranging from 2–4 weeks and one tissue which had been stored in formalin for two years were tested for immunoreactivity to vimentin and pan-cytokeratin. As shown in Table 4, without treatment only a minority of the tissues were stained, and the staining that did occur was usually weak. However, following antigen retrieval with lead solution, immunoreactivity for these two antibodies was significantly enhanced, indicating that retrieval of antigen in long-term formalin fixed tissues was possible.

Effect of Formalin Fixation on Formalin Sensitive Antigens

A single sample of malignant melanoma was divided into several pieces, and each piece was fixed in formalin for 22 hours at temperatures of 4° C., 25° C., or 37° C. As the process of formalin fixation is temperature dependent, higher temperatures produce more rapid fixation (5). Following paraffin embedding, these tissues were subjected to immunostaining for vimentin. Because the epitope recognized by this vimentin antibody (clone V9) is partially formalin sensitive (27), this system was used to investigate whether antigen retrieval could be used to reverse the deleterious effects of fixation in formalin. As shown in Table 5, the observed decrease in vimentin immunoreactivity was directly related to an increase in temperature of the formalin fixative. However, following antigen retrieval in the presence of lead solution, vimentin immunostaining was completely restored to a level even surpassing that observed in tumor fixed at 4° C. without subsequent treatment (Table 5). These results indicate that, at least for some epitopes, the deleterious effects of formalin fixation are reversible.

Controls

For most antibodies diluted to their optimal titer, the signal-to-noise ratio was usually much better with tissues treated for antigen retrieval compared to untreated tissues, as the background was usually lower following antigen retrieval. However, in some tissues which were already prone to high background (staining in the absence of primary antibody), treatment by antigen retrieval further enhanced background staining. This type of background was associated with the direct binding of the secondary biotinylated antibody to the tissue and could usually be eliminated by appropriate dilution of the secondary antibody.

Alcohol Fixation

When antigen retrieval was performed on sections of tissues fixed by alcohol, there was no enhancement of immunoreactivity, whereas all sections fixed in 10% formalin, irrespective of the length of time of fixation, showed increased immunoreactivity.

Microwave oven treatment had no observable effects on tissue morphology when viewed by light microscopy.

TABLE 2

Comparison of Conventional Heat to Microwave Heat for Antigen Retrieval in Overfixed Tissues

		CONVENTIONAL OVEN				MICROWAVE OVEN (Boiling)	
	Number of	80° C.		Boiling			
Antibody[a]	Tissues Tested	H_2O	Lead	H_2O	Lead	H_2O	Lead
CK 8, 18, 19	2	–[b]	+	++	+++	+++	+++++
IgD	2	+	++	++	+++	++++	+++++
Pan-CK	39	+	++	+++	++++	++++	+++++
Vimentin	39	+	++	+++	++++	++++	+++++

[a]Abbreviations used:
CK = Cytokeratin
[b]Immunoreactivity was scored on a scale of – to +++++. The reactivity score was an average value over all the tissues tested.

TABLE 3

A Comparison of Immunostaining Results Using Diluted Primary Antibodies

			Microwave[a] Oven				Trypsin
Antibody Dilution	Tissues	Non-Treatment	DW	Zn	With Lead	NC[b]	Digestion
Pan-K[c]	Tonsil	–[d]	++	+++	+++	–	–
CK AE1	Tonsil	–	+	++	+++	–	–
CK AE3	Tonsil	–	++	+++	+++	–	–
CK AE8	Tonsil	–	+	++	+++	–	–
CK 7	Adenocarcinoma[e]	–	++	++	+++	–	+/–
IgD	Tonsil	–	++	+	+++	–	–

[a]Microwave oven with:
DW = distilled water,
Zn = Zinc sulfate solution,
Lead = lead thiocyanate
[b]NC: Negative control or PBS was used to replace primary antibody on the slide treated by microwave oven with metal solution or distilled water.
[c]CK = cytokeratin
[d]Immunoreactivity was scored on a scale of – to +++. The reactivity score was an average value taken over the entire tissue.
[e]Adenocarcinoma, breast adenocarcinoma.

TABLE 4

Immunoreactivity of Long-Term Formalin Fixed Tissues[a]

	No Treatment		Microwave + Lead	
Antibody	Antibody	NC[b]	Antibody	NC
Vimentin	6/40[c]	0/40	40/40	0/40

TABLE 4-continued

Immunoreactivity of Long-Term Formalin Fixed Tissues[a]

	No Treatment		Microwave + Lead	
Antibody	Antibody	NC[b]	Antibody	NC
Pan-CK[e]	5/40	0/40	26/40[d]	0/40

[a]Staining method was by the MultiLink alkaline phosphatase system.
[b]NC = Nonimmune ascites negative control.
[c]Represents the number of tissues staining positive (+ to +++) over the total number of the tissues treated.
[d]Of the 40 tissues tested only 26 contained epithelial cells which would be positive for kerative staining.
[e]CK = cytokeratin.

TABLE 5

Effect of Fixation Temperature on Vimentin Immunoreactivity in Malignant Melanoma

Fixation[a]	No Treatment		Microwave + Lead	
Temperature	Vimentin	NC[b]	Vimentin	NC
4° C.	++[c]	–	+++	–
Pan-CK	5/40	–	+++	–
37° C.	–	–	+++	–

[a]Fixation in 10% neutral buffered formalin for 22 hours
[b]NC = nonimmune ascites negative control
[c]Immunoreactivity scored on a scale of – to +++

All publications and patent applications mentioned in this specification are herein incorporated by reference both the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

The invention now being fully described, it will be apparent to one of ordinary skill in the t than many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

What is claimed is:

1. A method for the preparation of formalin-fixed tissue for immunological staining, which comprises:

heating a formalin-fixed tissue preparation submersed in water with microwave energy for a time sufficient to increase immunostaining of said preparation in relation to immunostaining in the absence of said heating.

2. The method of claim 1, wherein said time is sufficient to boil said water.

3. The method of claim 1, wherein said water is an aqueous solution of a metal salt.

4. The method of claim 3, wherein said metal salt is a salt of lead or zinc.

5. The method of claim 2, wherein said water contains a dissolved metal salt and boils at a temperature at or above 100° C.

6. The method of claim 1, wherein said microwave energy has a frequency of from 1 to 50 GHz.

7. The method of claim 2, wherein said microwave energy is applied at a rate sufficient to cause 50 ml of said water to increase in temperature at a rate of from 0.5° to 5.0° C. per second prior to boiling.

8. The method of claim 2, wherein said boiling continues for up to 20 minutes.

9. The method of claim 3, wherein said method further comprises removing said tissue preparation from said water after being subjected to said microwave energy and washing to remove said aqueous solution.

10. A method of immunologically staining a formalin-fixed tissue preparation, which comprises:

(a) subjecting a formalin-fixed tissue preparation or tissue section on a microscope slide to microwave energy while said tissue preparation is submersed in water for a time sufficient to boil said water;

(b) removing said tissue preparation from said water and cooling to a temperature below 100° C.; and

(c) contacting said tissue preparation with an immunological staining reagent or series of reagents.

11. The method of claim 10, wherein said water is an aqueous solution of a metal salt.

12. The method of claim 11, wherein said metal salt is a salt of lead or zinc.

13. The method of claim 11, wherein said water contains a dissolved metal salt and boils at a temperature above 100° C.

14. The method of claim 10, wherein said microwave energy has a frequency of from 1 to 50 MHz.

15. The method of claim 10, wherein said microwave energy is applied at a rate sufficient to cause 50 ml of said water to increase in temperature at a rate of from 0.5° to 5° C. per second prior to boiling.

16. The method of claim 10, wherein said boiling continues for up to 20 minutes.

17. The method of claim 10, wherein said tissue preparation is stained with an enzyme-labelled monoclonal antibody.

18. The method of claim 10, wherein said tissue preparation is first reacted with an unlabeled monoclonal or polyclonal antibody and subsequently reacted with a reagent or series of reagents to introduce an enzyme label at the site of the antigen-antibody reaction.

* * * * *

EXHIBIT C

US006451551B1

(12) **United States Patent**
Zhan et al.

(10) **Patent No.: US 6,451,551 B1**
(45) **Date of Patent: Sep. 17, 2002**

(54) **RELEASING EMBEDDING MEDIA FROM TISSUE SPECIMENS**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/573,073**

(22) Filed: **May 16, 2000**

Related U.S. Application Data

(63) Continuation of application No. 08/212,175, filed on Mar. 11, 1994.

(51) **Int. Cl.**[7] **G01N 1/30**
(52) **U.S. Cl.** **435/40.52**; 435/40.5
(58) **Field of Search** 435/40.52, 40.5; 83/856, 915.5; 422/61; 427/2.11; 436/174, 176

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(57) **ABSTRACT**

Releasing the embedding medium from an embedded histochemically reactive tissue specimen is provided by contacting the embedded tissue specimen with a releasing composition under conditions sufficient to release a sufficient portion of the embedding medium associated with the histochemically reactive tissue specimen to permit analysis without substantial adverse effect on the histochemical reactivity of the specimen.

20 Claims, No Drawings

RELEASING EMBEDDING MEDIA FROM TISSUE SPECIMENS

This application is a continuation of Ser. No. 08/212,175 filed Mar. 11, 1994.

TECHNICAL FIELD

The present invention relates to methods and compositions for releasing embedded tissue specimens from the embedding medium.

BACKGROUND OF THE INVENTION

Paraffin has been used for many years as an embedding medium in techniques for the preparation of tissue specimens for sectioning in a microtome to produce specimen sections for histological studies. Such embedding techniques generally include the well known steps of specimen fixation, dehydration, clearing, paraffin infiltration or impregnation, blocking or embedding in a block of paraffin, slicing the block and specimen into thin sections, mounting the sections on slides, removing the paraffin and solvents employed for this purpose (commonly termed "deparaffinizing"), rehydration of tissue sections and staining the sections prior to analysis.

The primary purpose of the embedding medium is to permit the specimens to be sectioned and mounted in an approximation of the natural state. Plastic resins have also been used as embedding medium to provide a harder specimen that allows the cutting of thinner sections. However, the use of paraffin-embedding has the advantage that the wax can be dissolved away from specimens prior to staining, allowing sections to be stained in the form of naked slabs of biopolymer and avoiding the extra difficulties and artifacts associated with the presence of unremovable resin-embedding medium (Horobin, R. W., In "*Histochemical and Immunochemical Techniques: Application to pharmacology and toxicology*" (1991) Bach, P. and Baker, J., eds., Chapman & Hall, New York, N.Y., pp. 1–9).

Recent improvements in paraffin-embedding compositions have broadened the applicability of the technique while maintaining its compatibility with downstream manipulation and analysis of samples. For example, an improved paraffin-based embedding material, which includes a mixture of paraffin and an effective amount of ethylene-vinyl acetate copolymer (0.5% to 5% by weight of paraffin) is reported to allow shorter infiltration time and thinner sections (U.S. Pat. No. 4,497,792). Another improvement, the double-embedding technique, yields sections of tissue membranes that usually measure only 10 microns in thickness. In this method, several membranes are fixed and mounted on needles located at the bottom of a plastic box and then embedded in agarose. The agarose block is removed, dehydrated in alcohol, cleared with HistoPetrol (trade name for a mixture of isoparaffin hydrocarbons), permeated with paraffin and sectioned. The observed tissue morphology is comparable to that obtained with methacrylate plastic embedding but is less time-consuming, less hazardous since no plastic hardener and activator are used, and makes immunohistochemical studies easier (Ghassemifar, R. et al. "A double-embedding technique for thin tissue membranes" *Biotech. Histochem.* 67:363–366 (1992)). Consequently, deparaffmization of fixed, e.g. formalin fixed, paraffin-embedded tissue sections is still a widely used methodology, particularly in hospital histopathology laboratories for immunodiagnostic purposes.

Xylene, which is a flammable, volatile and toxic organic solvent, is commonly used in protocols to solubilize paraffin for deparaffinization of specimen sections. Typically, the microscope slide-mounted specimen is immersed in a xylene bath until the paraffin is solubilized. The treated specimen is then washed with a series of alcohol solutions of decreasing alcohol concentration, typically as baths in which the specimen is immersed, to remove xylene before a final wash with water. Efforts have been made to replace xylene in the deparaffinization technique with less toxic and less volatile solvents (Mullin, L. S. et al. "Toxicology update isoparaffmic hydrocarbons: A summary of physical properties, toxicity studies and human exposure data" *J. Appl. Toxicol.* 10:135–142 (1990)). Terpene oil (e.g. available under the trade name AmeriClear from Baxter Health Care Diagnostics, Inc., McGaw Park, Ill.) and isoparaffinic hydrocarbons (e.g. available under the trade name Micro-Clear from Micron Diagnostics, Inc., Fairfax, Va.) produced equal deparaffinization compared to xylene (Jones, R. T. et al. "Comparison of deparaffinization agents for an automated immunostainer" *J. Histotechnology* 16:367–369 (1993)). However, a series of alcohol washes were still required to remove either solvent prior to the water wash to achieve compatibility with most types of staining, particularly immunohistochemical staining.

Furthermore, the use of paraffin-embedded specimens with automated systems, such as automated immunostaining devices, is increasing. In these applications, the complexity of the multiple manipulations necessitated by conventional deparaffinization methodology creates a substantial obstacle to the efficient, cost-effective and reproducible handling of embedded tissue specimens.

Accordingly, there remains a need for compositions and methods that can effectively remove, or otherwise eliminate, paraffin, improved paraffin-based and other embedding materials from specimens prior to histochemical or other diagnostic analyses, while minimizing danger to users, allowing compatibility with automated systems, and maintaining compatibility with downstream analyses. Compositions and methods that entail no or limited toxicity or carcinogenicity, produce no or minimal odors, reduce the quantity of toxic solvents used, minimize hazardous wastes, and/or decrease corrosiveness and inflammability are desirable. One such composition and method which has found use is disclosed in PCT Publication WO95/24498, published on Sep. 14, 1995. However, it remains desirable to minimize the use of organic solvents, even those having minimal toxicity or carcinogenicity, odors, hazardous waste concerns, corrosiveness and inflammability.

DISCLOSURE OF THE INVENTION

The present invention provides methods and compositions for releasing the embedding medium from embedded histochemically reactive tissue specimens prior to histochemical or other analyses. In one aspect, the invention provides a method comprising contacting the embedded tissue specimen with a releasing composition comprising a non-polar organic solvent, a polar organic solvent, a surfactant, and water, under conditions sufficient to release a sufficient portion of the embedding medium associated with the histochemically reactive tissue specimen to permit analysis without substantial adverse effect on the histochemical reactivity of the specimen.

The methods provided can effectively remove or otherwise eliminate embedding media, and particularly wax or modified wax-based embedding media, more particularly paraffin or paraffin-based media, from tissue specimens prior to histochemical or other analyses, while minimizing danger

to users, allowing compatibility with automated use, and maintaining compatibility with downstream analyses. In this regard, it is considered important to release a portion of the embedding medium associated with the tissue specimen without substantial adverse effect on the histochemical reactivity of the specimen.

The present methods entail no known toxicity or carcinogenicity, no noxious or toxic odors, reduce the quantity of toxic solvents used, minimize hazardous wastes, and/or decrease corrosiveness and inflammability. The methods are especially useful for eliminating the use of xylene and for reducing the use of alcohol in preparation of tissue sections for histochemical staining, particularly in hospital laboratories. Compositions and kits for releasing the embedding medium from an embedded specimen are also provided. The composition comprises a non-polar organic solvent, a polar organic solvent, a surfactant, and water, and the kit comprises a releasing composition of the invention and a second composition of (1) a histochemical staining reagent or (2) an aqueous wash solution for removing, or otherwise eliminating, residual releasing solution.

Other aspects of the present invention will be readily apparent from the following more detailed description.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides methods and compositions for releasing the embedding medium from embedded histochemically reactive tissue specimens prior to histochemical or other analyses such as immunohistochemistry and in situ hybridization, special stains and classical dye stains. In one aspect, the invention provides a method comprising contacting the embedded tissue specimen with a releasing composition comprising a non-polar organic solvent, a polar organic solvent, a surfactant, and water, under conditions sufficient to release a sufficient portion of the embedding medium associated with the histochemically reactive tissue specimen to permit analysis without substantial adverse effect on the histochemical reactivity of the specimen.

The present invention eliminates or minimizes the use of xylene or xylene-substitute solvents in histological laboratories. The compositions and methodology described herein effectively remove, or otherwise eliminate, paraffin or other wax residue from tissue sections and have no adverse effect on the quality or histochemical reactivity of tissue sections prepared for histochemistry and in situ hybridization. Application of this methodology can be extended to other analytical applications where removal of embedding medium from tissue sections are desired, such as in situ hybridization, classical dye stains and special stains.

In one aspect, the present invention employs new releasing compositions for releasing embedding media, and particularly wax or modified wax-based embedding media, particularly paraffin or paraffin-based, from tissue specimens prior to histochemical or other analyses, while minimizing danger to users, allowing compatibility with automated use, and maintaining compatibility with downstream analyses. In this regard, it is considered important to release a portion of the embedding medium associated with the tissue specimen without substantial adverse effect on the histochemical reactivity of the specimen. In further embodiments the composition of the invention may optionally be diluted with water.

By "embedding medium" is meant any composition that is used in the histochemical art for embedding or otherwise supporting histochemically reactive tissue specimens for histochemical or other analyses, such as immunohistochemistry and in situ hybridization, special stains and classical dye stains. As one example of an embedding medium, wax is often used for this purpose.

By "wax" is meant a composition used in the histochemical art for embedding histochemically reactive specimens for histochemical or other analyses that is typically solid at room temperature, usually consists of a complex mixture of higher hydrocarbons often including esters of higher fatty acids and higher glycols, can be mineral, natural or synthetic in origin, is harder and more brittle than fats, is soluble in oils and fats, and can optionally contain additives that enhance its specimen-embedding properties. Paraffin is an example of a mineral wax most commonly used in the histochemical field. Paraffin is typically prepared by distillation of petroleum, and is a mixture of primarily solid saturated hydrocarbons.

By "histochemical" is meant generally the chemical analysis of tissue specimens for morphological, genetic or other characteristics, and is meant to include, but not be limited to, the techniques and methods known as immunohistochemical, cytochemical, histopathologic, hematoxylin and eosin (H&E) staining, enzyme histochemical, special stain, micro technique, in situ hybridization, and the use of molecular probes. Texts illustrating histochemical techniques include "Histochemical and Immunochemical Techniques: Application to pharmacology and toxicology," (1991) Bach, P. and Baker, J., eds., Chapman & Hall, New York, N.Y. pp. 1–9, and in "Stains and Cytochemical Methods," (1993) M. A. Hayat, ed., Plenum Press, New York, N.Y.

By "releasing the embedding medium" is meant removing or otherwise eliminating a sufficient amount of the embedding medium associated with a tissue specimen so as to permit the histochemically reactive tissue specimen to be subjected to analysis. Typically, such analysis is histochemical, and the amount of the embedding medium which should be removed will be the amount sufficient to permit the analysis technique of choice to gain access to at least one of the histochemically reactive sites in the histochemically reactive tissue specimen.

By "histochemically reactive tissue specimen" is meant a sample of animal or plant cells or tissues which is selected and treated so as to preserve a detectable amount of the native histochemical reactivity inherent in the sampled organism prior to the sampling. Typically, such specimens are obtained as tissue sections by biopsy, necropsy, and the like, in accordance with techniques know in the histochemical arts.

Because the present compositions are typically prepared by combining components without a precise determination of the final volume of the composition or accounting for volume changes upon mixing, the percentages for each component are qualified with the term "about" or "approximately," with the understanding that one skilled in the art would appreciate the imprecision of the values as a consequence of composition preparation; however, preferably, percentage values are taken to mean their precise value when volume changes upon mixing are taken into account.

In accordance with the invention, the non-polar organic solvent is a hydrocarbon or mixture of hydrocarbons (e.g. as from a petroleum distillate) that has a boiling point well above room temperature, preferably above 110° C., more preferably from about 140° C. to about 250° C., that is in liquid phase at the temperatures used with the present

invention (usually 5° to 100° C.), and that is capable of dissolving or otherwise releasing the embedding medium used for embedding biological specimens. The present non-polar solvent can be a complex mixture of long-chain linear and branches alkane hydrocarbons containing, for example, esters of fatty acids and higher glycols. As a representative example for releasing an embedding medium, the paraffin solubility of the solvent at 25° C. is typically at least 0.1 gram of paraffin per liter of solvent, preferably 0.1 gram per 100 mL of solvent, more preferably 0.1 gram per 10 mL of solvent, and most preferably capable of a dissolving an amount of paraffin equal to about 50% of the solvent solutions weight. The non-polar solvent is further desirably miscible with a polar organic solvent when used in a composition of the invention. Examples of non-polar organic solvents include aromatic hydrocarbons, aliphatic hydrocarbons, terpenes, other oils, and petroleum distillates. Preferred non-polar organic solvents have little or no toxic effects. Furthermore preferred solvents are those not classified by the Environmental Protection Agency as hazardous waste. A preferred non-polar solvent furthermore has a flash point higher than about 60° C. which minimizes flanmmability. A preferred solvent also lacks carcinogenicity and corrosiveness. An isoparaffinic hydrocarbon is an example of a preferred non-polar solvent, in part because of its lack of toxicity, carcinogenicity, corrosiveness and flammability (Mullin et al. 1990). Preferred isoparaffins are branched aliphatic hydrocarbons with a carbon skeleton length ranging from approximately C_{10} to C_{15}, or mixtures thereof. One preferred isoparaffin hydrocarbon mixture has a flashpoint of about 74° C. Another preferred non-polar solvent is a mixture of C_{10} to C_{50} branched or linear hydrocarbon chains having a distillation range from a boiling point of 150° C. to about 250° C., and has the general formula of $C_nH_{(2n\pm m)}$ where n=10–50 and m=0–4. Mineral spirits is another preferred non-polar organic solvent. A preferred terpene is limonene. Other terpenes that can be used include terpinenes and terpineols. Less preferably, the solvent is an aromatic hydrocarbon solvent such as an alkyl benzene, e.g. xylene, or dialkylbenzene, e.g. toluene. Toluene and xylene are less preferred because of their toxicity and rating as hazardous waste. Furthermore, as discussed below, even when xylene or toluene are used in embodiments of the invention, subsequent alcohol washes are eliminated and replaced with a non-hazardous aqueous wash solution. In certain embodiments of the invention, the aqueous wash solution can simply include water, in alternative embodiments the solution will contain buffer, salts or other reagents useful for solubilization or releasing of the embedding medium, washes, or subsequent histochemical steps, so long as such optional ingredients or reagents do not interfere with the efficiency of releasing, a washing step, or subsequent histochemical steps.

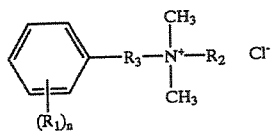
The non-polar organic solvent of the present composition is typically from about 1% to about 50% by volume of the releasing composition. Below the lower percent limit of non-polar organic solvent the capability of the composition to release an embedding medium is often significantly decreased. Above the upper limit of non-polar solvent an adverse affect on detergent solubility or water solubility occurs, which adversely affects the effectiveness of a subsequent aqueous wash. The upper limit of solvent can range through the upper limit values of 50 to 75%, while the lower limit of solvent can be selected from the lower limit values of 1 to 25%, to obtain a variety of ranges for embodiments of the invention.

The polar organic solvent of the present invention generally serves the purpose of dissolving the non-polar solvent, surfactant and, optionally, water. The polar organic solvent is soluble in water to the extent of at least 1 g per 100 g water, preferably 5 g per 100 g water, more preferably 10 g per 100 g water and most preferably the polar organic solvent is miscible with water. Polar organic solvents include ketones and lower alcohols, which include polyhydroxy alcohols and glycols, and lower ethers. Preferred alcohols are C_1 to C_5 alcohols. Most preferred are ethanol, ethylene glycol, isopropanol, propylene glycol and mixtures thereof. A preferred ketone solvent is typically C_3 to C_5 ketone. Most preferred ketone solvents are acetone and methyl ethyl ketone. Preferred ethers are C_2 to C_6 ethers. Particularly preferred polar organic solvents are selected from the group consisting of methanol, ethanol, isopropanol, butanol, tert-butanol, allyl alcohol, acetone, ethylene glycol and propylene glycol, and a mixture thereof. Acetonitrile and dimethylformamide are less preferred polar organic solvents. Furthermore, the polar organic solvent can be a mixture of polar organic solvents.

The polar organic solvent in the composition is typically from about 5% to about 50% by volume of the composition. The upper limit of this solvent can be selected from the range of upper limit values of 50 to 75%, while the lower limit of solvent can be selected from the range of lower limit values of 5 to 25%, to obtain a variety of ranges for embodiments of the invention. Preferably the amount is from about 10% to about 40%, more preferably from about 20% to about 35%, and most preferably from about 20% to about 30%. At what combination of components a particular composition is miscible or separates can readily be determined from a phase diagram showing phase separation for different relative amounts of the components of the solution/mixture.

Surfactants which find use in the present invention include cationic surfactants, anionic surfactants, non-ionic surfactants, and zwitterionic surfactants. A number of biological detergents (surfactants) are listed as such by Sigma Chemical Company in its catalog of Biochemicals and Reagents Life Science Research. The surfactant serves the purpose of a detergent, since it has both hydrophilic and hydrophobic properties. A surfactant for use in the invention is soluble in the solvent used in a composition of the invention. Preferred surfactants are detergents that are soluble in water, ethanol and acetone. Most preferred are those that do not substantially interfere with downstream histochemical analyses, which can be determined, for example, by histochemical staining using a solution containing the surfactant.

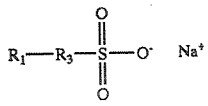
Surfactants that can be used in compositions of the invention include cationic surfactants of the formula



wherein R_1, is methyl, ethyl or propyl or isopropyl where n is 1 or 2; R_2, is an alkyl from CH_3 to $C_{30}H_{61}$ or a benzyl group; and R_3 is $(CH_2)_m$, where mn is from 1 to 10, or R_3 is (OCH_2CH_2), where p is from 1 to 10. Cationic surfactants of this formula are soluble in the polar organic solvents. Many preferred embodiments of the invention contain the cationic surfactant benzalkonium chloride or benzethonium chloride. Additional cationic detergents, not necessarily of this formula, include dodecyltrimethylammonium bromide,

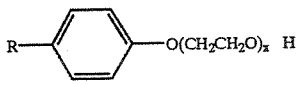
benzyldimethylhexadecyl ammnonium chloride, cetyiphyridinium chloride, methylbenzethonium chloride, and 4-picoline dodecyl sulfate.

Other surfactants that can be used in the compositions of the invention include anionic surfactants having the formula

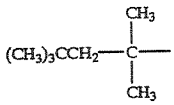


wherein R_1 is C_6H_{13} to $C_{30}H_{61}$, and R_3 is O, CH_2 or phenyl group. Anionic surfactants of this formula are soluble in a polar organic solvent. Examples of anionic detergents, not necessarily having this formula, include alginic acid, caprylic acid, cholic acid, 1-decanesulfonic acid, deoxycholic acid, 1-dodecanesulfonic acid, N-lauroylsarcosine, and taurocholic acid. Other anionic synthetic non-soap detergents, which are represented by the water-soluble salts of organic sulfuric acid reaction products, have in their molecular structure an alkyl radical containing from about 8 to 22 carbon atoms and a radical selected from the group consisting of sulfonic acid and sulfuric acid ester radicals. Examples of these are the sodium or potassium alkyl sulfates, derived from tallow or coconut oil; sodium or potassium alkyl benzene sulfonates; sodium alkyl glyceryl ester sulfonates; sodium coconut oil fatty acid monoglyceride sulfonates and sulfates; sodium or potassium sales of sulfuric acid esters of the reaction product of one mole of a higher fatty alcohol and about 1 to 6 moles of ethylene oxide per molecule and in which the alkyl radicals contain from 8 to 12 carbon atoms; the reaction product of fatty acids esterified with isethionic acid and neutralized with sodium hydroxide, sodium or potassium salts of fatty acid amide of a methyl tauride; and sodium and potassium salts of SO_3-sulfonated C_{10}–C_{24} α-olefins.

Further surfactants that can be used in compositions of the invention include non-ionic surfactants having the formula



wherein R is a linear or branched C1 to C10 alkyl group and X is an integer from 5 to 40. Most preferably R is



Non-ionic surfactants of this formula are soluble in polar organic solvents. Examples of nonionic detergents, not necessarily having this formula, include decanoyl-N-methylglucamide, diethylene glycol monopentyl ether, n-dodecyl P-D-glucopyranoside, polyoxyethylene esters of fatty acids (particularly C_{12}–C_{20} fatty acids, (e.g., sold under the trade name Triton), ethylene oxide condensates of fatty alcohols e.g. sold under the name Lubrol), polyoxyethylene sorbitan fatty acid esters (e.g., sold under the trade name Tween), and sorbitan fatty acid esters (e.g., sold under the trade name Span). Nonionic synthetic detergents made by the condensation of alkaline oxide groups with an organic hydrophobic compound. Typical hydrophobic groups include condensation products of propylene oxide with propylene glycol, alkyl phenols, condensation product of propylene oxide and ethylene diamine, aliphatic alcohols having 8 to 22 carbon atoms, and amides of fatty acids. Also nonionic detergents such as amine oxides, phosphine oxides and sulfoxides having semipolar characteristics and be removed. Specific examples of long chain tertiary amine oxides include dimethyldodecylamine oxide and bis-(2-hydroxyethyl)dodecylamine. Specific examples of phosphine oxides are found in U.S. Pat. No. 3,304,263, and include dimethyldodecylphosphine oxide and dimethyl-(2-hydroxydodecyl) phosphine oxide. A preferred non-ionic detergent surfactant is Triton X-100, which is a trade name for a polyoxyethylene ester of fatty acids (particularly C_{12}–C_{20} fatty acids).

Zwitterionic surfactants include known compounds of the formula N-alkyl-N, N, -dimethyl-3-ammonio-1-propanesulfonate. Examples of zwitterionic detergents include 3-[3-cholamidopropyl)-dimethylamrnmonio]-1] propanesulfonate (commonly abbreviated CHAPS), 3-[cholamidopropyl)-dimethylammonio]-2-hydroxy-1-propane sulfonate (generally abbreviated CHAPSO), N-dodecyl-N-dimethyl-3-ammonio-1-propane sulfonate, and lyso-α-phosphatidyl-choline.

The surfactant concentration in the composition of the present invention is typically from about 0.05% to about 50% by weight to volume, more commonly from about 0.5% to about 20% weight to volume, of the composition. Below the lower limit of surfactant concentration, poor solubility of wax (or releasing of the embedding medium) in the composition is observed. The upper limit of surfactant concentration is a primarily a factor of the selected surfactant's solubility limit.

Compositions of the invention can also contain water. Most preferably the amount of water in a selected composition is a saturating amount of water. Above this upper limit phase separation of the composition occurs. Because the compositions of the present invention can be used throughout a wide temperature range, the amount of water, and thus the concentrations of the remaining components of the composition, can vary widely. Typically the amount of water in the composition will range from about 0.5% to about 95% by volume. Where the composition is intended to be used at the lower end of the temperature range, the amount of water is less than about 30% and often less than about 10% by volume of the composition. Some embodiments of the invention, for example as exemplified in the Examples, have less than about 7% water, some have from about 0.5% to about 1.5% water, and still others have less than about 1% water by volume. Conversely, where the composition is intended to be used at the upper end of the temperature range, that is, at or near 100° C., the amount of water can be greater than about 50% and often greater than about 80% by volume of the composition. Some embodiments of the invention have more than about 70% water.

In some embodiments of the invention, the releasing compositions or the aqueous wash solutions contain buffer, salts or other reagents useful for solubilization of the embedding medium, washes, or subsequent histochernical steps, so long as such optional reagents do not interfere with the releasing capability of the composition, the efficiency of a washing step, or subsequent histochemical steps. Reagents useful for subsequent processing or histochemical steps include carboxylic acid esters, enzymes such as lipases, and nucleophilic reagents as described in U.S. Pat. No. 5,578,452, which is incorporated herein by reference. Optional agents can serve to expose or enhance aldehyde-fixed tissue antigen(s) for histochemical staining. Additional optional reagents include anti-microbial agents and stabilizers that

increase composition shelf life. Such anti-microbial agents and stabilizers are well known in the field. Such reagents are typically used at extremely small percentages, typically below 0. 1%, compared to the main components. Preferred reagents are those that do not interfere with downstream histochemical analyses.

Each of the individual components of the compositions of the invention is either commercially obtainable, is isolated from natural sources using known procedures, or is synthesized according to known procedures. Compositions of the invention are typically prepared bysimple mixing of the components in the indicated amounts.

Methods of preparing histochemically reactive samples for sectioning via wax-or paraffin-impregnation are generally well known and easily carried out. The technique is quite simple and involves contacting a wax-embedded specimen with a releasing composition of the invention to solubilize the wax that impregnates the specimen prior to histochemical analyses, such as immunostaining. The method optionally comprises a further step of contacting the treated specimen immediately after releasing with an aqueous washing composition comprising a detergent to remove residual releasing composition.

Although the releasing method is typically and conveniently carried out in a range approximating room temperature, without the need for a temperature controlled bath, a more precise control of the required time for satisfactory releasing and washing is available if temperature-controlled baths are used. Heating decreases processing time. Operable temperatures overall range from about 5° to the boiling temperature of the solution, preferably from about 15° C. to the boiling temperature of the solution. In the lower end of the range, the method can be performed from near room temperature to near physiologic temperature, that is from about 20° C. to about 40° C. In this temperature range, the compositions will typically be selected with non-aqueous components in the upper ranges specified previously. In the upper end of the temperature range, the method can be performed from above physiologic temperature up to and including the boiling temperature of the composition, that is from about 40° C. to about 100° C. (or actual boiling temperature). In this temperature range, the compositions will typically be selected with non-aqueous components in the lower ranges specified previously, and the water will typically form a major portion of the releasing composition.

Typically the embedded specimen is contacted with a composition of the invention for a time sufficient to solubilize or release all or part of the embedding medium associated with the specimen. Factors influencing the time required for satisfactory results include temperature, thickness of the specimen section and composition of the embedding medium. Time for any particular specimen type is ordinarily determined empirically. However, five minutes of contact is usually sufficient for specimens of standard thickness mounted on microscope slides.

A sectioned specimen, typically affixed to a microscope slide, is contacted with a composition of the invention in any number of ways. Preferably, the specimen is immersed in a bath containing the composition, or alternatively an amount of composition sufficient to solubilize the wax can be placed on the specimen such that the specimen is covered by the composition. After sufficient time of contact has elapsed for releasing to occur, the specimen is removed from contact with the composition, and excess composition is removed, or otherwise eliminated, from the specimen, for example by draining, blotting or blowing. Optionally, a second or even a further releasing step or steps are performed, preferably with fresh releasing composition, to further assure removal or elimination of wax from the specimen.

The invention decreases or eliminates the requirement of alcohol baths for post-releasing washes, and post-releasing washes are not always required with compositions of the invention. If such a step proves desirable (because of a particularly sensitive immunostaining procedure, for example) the treated specimen can be contacted with an aqueous wash composition of the invention which comprises a detergent. A preferred wash solution comprises a buffer and a detergent. Preferably the detergent is non-ionic. A preferred buffer/detergent wash solution is phosphate buffered saline with about 1% nonionic surfactant polyoxyethylene ester such as BRIJ-35 (trade name for the nonionic surfactant polyoxyethylene glycol dodecyl ester or polyoxyethylene (23) lauryl ester). Typically the amount of detergent is from about 0. 1% to about 5% (weight to volume), preferably from about 0.1% to 2%, and most preferably about 1%. The pH of the wash composition can range from about 2 to about 12, preferably from about 5 to about 8, more preferably from about 7.2 to about 7.6, and more preferably 7.4 to about 7.5. The pH is most preferably neutral to avoid adversely affecting downstream histochemical, particularly imniunochemical, analyses.

A preferred buffer is one which does not interfere with downstream analyses and/or can be readily removed with a subsequent aqueous wash or blowing. Phosphate buffered saline or Tris-containing buffers are examples of preferred buffers. Washing can occur in any number of ways, including immersion in a wash bath, flowing wash solution over the specimen, diffusing or permeating the wash solution throughout the specimen, or blowing. Wash time is ordinarily determined empirically; however, five minutes is usually sufficient. Multiple rinses and larger amounts of washing solution can be used to achieve increased removal of releasing solution. A single wash is sufficient for most purposes; however, a second wash is preferred if removal is not sufficient. Optionally, the specimen is finally washed or rinsed in water. A water wash of 3 minutes is usually sufficient for the most rigorous conditions. After washing the specimen is then ready for histochemical or other analyses.

The compositions of the invention, including the wash solutions, are also compatible with automated staining systems and devices, as described, for example, in U.S. Pat. Nos. 5,439,649 and 5,948,359, each of which are hereby incorporated by reference. Typically, such devices will comprise means for performing a predetermined sequence of operations under a predetermined set of conditions for histochemical analysis. Representative of such a device and system is the OptiMax™ Automated Immunostainer, BioGenex Laboratories San Ramon, Calif. In such automated histochemical analysis, previously treated slides can be provided to the automated stainer or an automated stainer can be provided with compositions of the invention to allow automated releasing of the embedded specimens prior to automated analyses.

Although preferred surfactants and other components used in a releasing solution of the invention are those that do not typically interfere with downstream analyses, particularly at the residual levels remaining on the specimen after the wash procedures, methods known in the art may be applied to enhance surfactant (or other component) removal should residual surfactant (or other component) cause problems in downstream analyses. For residual surfactant removal soluble compounds known to bind a surfactant may be included in an aqueous wash solution. For example,

at 25° C., the releasing composition is decanted and replaced with fresh releasing composition and the slides are treated for an additional five minutes.

Optionally, a third five minute releasing treatment can also be performed. Immediately after releasing, slides are rinsed in an aqueous wash composition containing PBS with 1% BRIJ-35 for five minutes, rinsed in tap water for three minutes, and used for histochemical analysis.

Example 2B

Releasing of Embedding Media at High Temperature

Each slide containing slide-mounted, paraffin-embedded tissue sections is immersed in a glassjar containing 60 mL of one of the releasing Compositions 7–8. After five minutes at 100° C., the releasing composition is decanted. Optionally, a second or third five minute releasing treatment can also be performed. Immediately after releasing, slides are rinsed in an aqueous wash composition containing PBS with 1% BRIJ-35 for five minutes, rinsed in tap water for three minutes, and used for manual or automated histochemical analysis.

Example 3

Automated Releasing of Embedding Media

Releasing embedding media from slide-mounted tissue specimens using Compositions 1–6 of Example 1 individually is performed as one phase of automated histochemical analysis, generally as follows. Human or animal tissues used in this Example include skin, pancreas, tonsil, spleen, lung, breast prostate, colon carcinoma, melanoma and astrocytoma.

Each slide containing slide-mounted, paraffin-embedded tissue sections is loaded onto a slide rack of an automated, consolidated histochemical staining apparatus (e.g. OptiMax™ Automated Immunostainer, BioGenex Laboratories San Ramon, Calif.) and the releasing procedure is implemented in accordance with the instructions of the manufacturer. In this regard, the reagent-dispensing head dispenses 2 to 3 mL of one of the releasing Compositions 1–6 on each slide. The slide-mounted tissue sections are incubated with the releasing composition for three minutes, the releasing composition is removed by the air orifice with blowing action and, without a rinse phase, replaced with 2 to 3 mL of fresh releasing composition for a further three minutes.

Thereafter, the releasing composition is again removed by the air orifice, and the slides are subjected to a series of wash solution rinses, first with one to three cycles of deionized water, then with one to three cycles of a buffer solution.

Typically, the settings for cycles and incubation times are selected as the default settings in accordance with the protocols established by the manufacturer; alternatively, the number and durations of the incubations and rinse cycles can be adjusted according to the preference of the user.

Example 4

Releasing with Xylene

A widely used, standard deparaffinization protocol involving xylene is performed as a control. Slide-mounted, paraffin-embedded tissue specimens are immersed in 100% xylene for five minutes followed by two changes in fresh 100% xylene for five minutes each.

Thereafter, the slides are immersed in a bath of 100% alcohol twice for three rminutes each time. The slides are then immersed sequentially in baths of 95% alcohol, 85% alcohol and then 75% alcohol for three minutes in each bath. The slides are finally rinsed in tap water for three minutes and used for histochemical analysis.

A further series of slides are prepared following this protocol, but substituting either limonene or Micro-Clear for xylene.

Example 5

Effectiveness of Paraffin Removal

The released slides prepared as in Examples 2, 3 and 4 are examined after hematoxylin and eosin (H&E) staining for effectiveness of paraffin removal or elimination. After the slides are released with releasing Compositions 1–6 and washed with aqueous wash composition, no paraffin residue is detected on the specimens or on other locations on the slides. In addition, no paraffin residue is detected on specimens or slides cleared with the control procedure using xylene and hydrated with graded alcohols and water followed by H&E staining. There is no discernible difference in effectiveness for paraffin removal among xylene, limonene, isoparaffin, and releasing Compositions 1–8 of Example 1.

Example 6

Effect of Releasing Solvents on Immunohistochemistry Staining

Normal or tumorous animal tissues including skin, pancreas, tonsil, spleen, lung, breast, prostate, colon carcinoma, melanoma and astrocytoma, are stained with corresponding monoclonal antibodies to determine the effects of the present releasing compositions on immunohistochemical staining. Xylene treated tissue specimen slides are used as standard controls.

Slides containing tissue specimens released as described in Examples 2 and 3 are examined for compatibility to immunohistological analyses. Released slides are covered in Block Solution I (a trade name of BioGenex Laboratories, San Ramon, Calif., for a solution of PBS and 3% hydrogen peroxide) for ten minutes. Each slide is then rinsed in PBS twice, for five minutes each time. Primary antibodies (200 μL; obtained from BioGenex Laboratories, San Ramon, Calif., under the trade name Ready to Use Antibodies) are incubated with their respective tissue specimens for 30 minutes or two hours, according to individual staining protocols provided by the supplier. The following monoclonal antibodies are used in immnunohistochemistry: anti-human cytokeratin cocktail, anti-NSE, anti-insulin, anti-LCA, anti-kappa chain, anti-Q-band, anti-L26, anti-factor VIII, anti-CEA, anti-p53, anti-Cerb-2, anti-PR, anti-vimentin, anti-PSA, anti-HMB45, anti-S-100 and anti-GFAP. The slides are then washed in PBS three times, for five minutes each time. After a 20 minute incubation with biotinylated secondary antibodies (available under the trade name Super Sensitive Link from BioGenex Laboratories, San Ramon, Calif.), the slides are washed in PBS three times, for five minutes each time. The slides are then incubated with a stock solution of peroxidase-conjugated streptavidin (available under the trade name Super Sensitive Label from BioGenex Laboratories, San Ramon, Calif.) for 20 minutes and washed three times in PBS. Desirably, the slides can be stained utilizing stains fro BioGenex Laboratories in the OptiMaxM Plus system. The chromogenic

cyclodextrins are known to bind certain surfactants (U.S. Pat. No. 5,032,503) and may be included in a wash solution. Protein, such as bovine serum albumin, can be included in a wash solution to bind and remove residual surfactant. In one preferred embodiment, a surfactant that does not interfere with the downstream analyses, but that can displace the residual surfactant, can be used in an aqueous wash solution. This displacing surfactant is preferably easily removed with a water wash. Polyoxyethylene alkyl ester type non-ionic surfactants are a preferred wash surfactant. BRIJ-35 (trade name for polyoxyethylene glycol dodecyl ester) is an example of one such surfactant.

Also provided is a kit for releasing the embedding medium from an embedded specimen. The kit comprises a container of releasing composition and containers of (1) histochemically reactive staining reagents or (2) an aqueous wash solution for removing, or otherwise eliminating, residual releasing solution. The containers are typically located in a receptacle specifically adapted to hold them. Preferably the wash solution contains a buffer and a detergent. In one embodiment the histochemically reactive staining reagent is an immunostaining reagent. In another embodiment the histochemically reactive staining reagent is an in situ hybridization reagent. The kit can be a component of a larger kit for histochemical analyses, such as in a kit for use with automated immnunostainers. Any of the other reagents described herein can be used in the kit in combination with the specified components.

The compositions and methods of the invention are suitable for use in a variety of histochemical applications, particularly immunochemical staining using special stains and other classical stains. In situ hybridization with nucleic acid probes is another particularly pertinent use compatible with compositions and methods of the invention.

The present invention eliminates or reduces the use of certain toxic organic solvents (e.g. xylene, xylene substitutes, alcohols, and the like) in immunohistologicai laboratories. The compositions and methodology described herein effectively removes, or otherwise eliminates, paraffin and other waxes residues from tissue sections and has no adverse effect on the quality of tissue sections prepared for histochemical analysis. Application of this releasing methodology can be extended to other applications where removal of paraffin and other waxes from tissue sections are necessary. In preferred embodiments using isoparaffins; the compositions have a very low order of acute toxicity, being practically non-toxic by oral, dermal and inhalation routes. In addition the compositions allow a method of releasing that eliminates the use of graded alcohol washes. Accordingly, the embodiments of the present invention meet the need of providing compositions and methods that minimize dangers to the user and minimize the creation of hazardous waste.

The invention now being generally described, the same will be better understood by reference to the following detailed examples which are provided for illustration and are not to be considered as limiting the invention unless so specified.

EXPERIMENTAL

In the experimental disclosure which follows, all weights are given in grams (g), milligrams (mg), micrograms (μg), nanograms (ng), or picograms (pg), all amounts are given in moles (mol), millimoles (mmol), micromoles (μmol), nanomoles (nmol), picomoles (pmol), or femtomoles (fmol), all concentrations are given as percent by volume (%), proportion by volume (v:v), molar (M), millimolar (mM), micromolar (μM), nanomolar (nM), picomolar (pM), femtomolar (fM), or normal (N), all volumes are given in liters (L), milliliters (mL), or microliters (μL), and linear measurements are given in millimeters (mm), or nanometers (nm) unless otherwise indicated.

The following examples demonstrate the practice of the present invention in preparing embedded tissue specimens for histochemical analyses.

Example 1

Releasing Compositions

The following examples of releasing compositions are presented by way of illustration of embodiments of the invention and not intended to limit the invention.

Composition 1 is prepared by. mixing reagent alcohol (275 mL; a premixed solution of 90% v/v anhydrous ethyl alcohol, 5% v/v methyl alcohol and 5% v/v isopropyl alcohol), limonene (100 mL), water (25 mL) and benzalkonium (20 g).

Composition 2 is prepared by mixing. reagent alcohol (50 mL), limonene (50 mL) and benzalkonium (10 g).

Composition 3 is prepared by mixing reagent alcohol (50 mL), isoparaffin (50mL), water (0.6 mL) and benzalkonium (15 g).

Composition 4 is prepared by mixing reagent alcohol (100 mL), isoparaffin (50 mL), mineral spirits (50 mL) and benzalkonium (15 g).

Composition 5 is prepared by mixing reagent alcohol (50 mL), isoparaffin (50 mL), water (0.9 mL) and Triton-X100 (10 g).

Composition 6 is prepared by mixing reagent alcohol (65 mL), isoparaffin (45 mL), water (0.5 mL) and BRIJ-35 (1.0 g).

Composition 7 is prepared by mixing citric acid (0.48 g), reagent alcohol (20 mL), isoparaffin (20 mL), water (1000 mL) and Triton-X100 (10 mL), then adjusting the pH to 8.5 with 20% NaOH solution.

Composition 8 is prepared by mixing citric acid (0.48 g), reagent alcohol (40 mL), water (1000 mL), Triton-X100 (10 mL), and BRIJ-35 (1.0 g), then adjusting the pH to 7.4 with 20% NaOH solution.

One embodiment of the isoparaffin (isoparaffinic hydrocarbon solvent) used in the compositions of this example is available as Micro-Clear, a trade name of Micron Diagnostics, Inc., for its isoparaffinic hydrocarbon solvent.

Example 2

Manual Releasing of Embedding Media

Releasing embedding media from slidemounted tissue specimens using each of Compositions 1–8 of Example 1 individually is performed prior to histological analysis. Humain or animal tissues used in this Example include skin, pancreas, tonsil, spleen, lung, breast prostate, colon carcinoma, melanoma and astrocytoma.

Example 2A

Releasing of Embedding Media at Low Temperature

Each slide containing slide-mounted, paraffin-embedded tissue sections is immersed in a glass-jar containing 60 mL of one of the releasing Compositions 1–6. After five minutes

reaction is carried out using AEC (3-amino-9-ethylcarbozole) for peroxidase and Fast Red for alkaline phosphatase. After color development, each slide is rinsed in tap water, counter-stained, mounted and examined by light microscopy.

Intensity of immunostaining reactivity is evaluated by a light-microscope. There is found to be no detectable difference in immunostaining intensity among slide-mounted specimens released with releasing Compositions 1–8, and specimens treated with the control with xylene.

All patents and patent applications cited in this specification are hereby incorporated by reference as if they had been specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent to those of ordinary skill in the art in light of the disclosure that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

What is claimed is:

1. A method for releasing the embedding medium from an embedded histochemically reactive tissue specimen, said method comprising contacting said embedded tissue specimen with a releasing composition comprising a non-polar organic solvent, a polar organic solvent, a surfactant, and water, under conditions sufficient to release a sufficient portion of the embedding medium associated with the histochemically reactive tissue specimen to permit analysis without substantial adverse effect on the histochemical reactivity of the specimen.

2. The method of claim 1 wherein the conditions for contacting said embedded tissue specimen comprise incubating said composition at a temperature in the range of approximately 10° C. to approximately 100 ° C. for a time sufficient to release a substantial portion of the embedding medium associated with the tissue specimen.

3. The method of claim 1 which further comprises the step of washing said tissue specimen after said contacting step with an aqueous wash solution under conditions sufficient to remove, or otherwise eliminate, at least a portion of any residual releasing composition from said tissue specimen.

4. An automated method for releasing the embedding medium from an embedded histochemically reactive tissue specimen, said automated method comprising

(a) providing an apparatus comprising means for performing a predetermined sequence of operations under a predetermined set of conditions for histochemical analysis which includes at least the step of releasing the embedding medium from an embedded histochemically reactive tissue specimen;

and

(b) contacting said embedded tissue specimen under the control of said apparatus with a releasing composition comprising a non-polar organic solvent, a polar organic solvent, a surfactant, and water, under conditions sufficient to release a sufficient portion of the embedding medium associated with the histochemically reactive tissue specimen to permit analysis without substantial adverse effect on the histochemical reactivity of the specimen.

5. The method of claim 4 wherein the conditions imposed by said apparatus for contacting said embedded tissue specimen comprise incubating said composition at a temperature in the range of approximately 10° C. to approximately 100° C. for a time sufficient to release a substantial portion of the embedding medium associated with the tissue specimen.

6. The method of claim 4 which further comprises the step of washing said tissue specimen under the control of said apparatus after said contacting step with an aqueous wash solution under conditions sufficient to remove, or otherwise eliminate, at least a portion of any residual releasing composition from said tissue specimen.

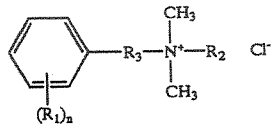
7. The method of claim 1, wherein the non-polar organic solvent comprises at least one aromatic hydrocarbon, terpene or isopararaffinic hydrocarbon.

8. The method of claim 1, wherein the non-polar organic solvent is from about 1% to about 50% by volume of said composition.

9. The method of claim 1, wherein the polar organic solvent comprises at least one alcohol, ketone, or other.

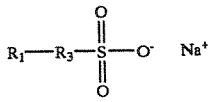
10. The method of claim 1, wherein the polar organic solvent is from about 1% to about 50% by volume of said composition.

11. The method of claim 1, wherein the surfactant comprises at least one cationic surfactant having the formula



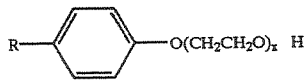
wherein R_1 is methyl, ethyl or propyl or isopropyl where n is 1 or 2; R_2 is an alkyl from CH_2 to $C_{30}H_{61}$ or benzyl group; and R_3 is $(CH_2)_m$ where m is from 1 to 10, or R_3 is $(OCH_2CH_2)_p$ where p is from 1 to 10.

12. The method of claim 1, wherein the surfactant comprises at least on anionic surfactant having the formula



wherein R_1 is C_6H_{11} to $C_{30}H_{61}$ and R_3 is CH_2 or a phenyl group.

13. The method of claim 1, wherein the surfactant comprises at least one non-ionic surfactant having the formula



wherein R is a linear or branched C1 to C10 alkyl group and X is from 5 to 40.

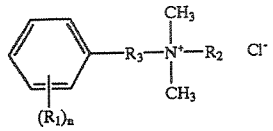
14. The method of claim 4, wherein the non-polar organic solvent comprises at least one aromatic hydrocarbon, terpene or isoparaffinic hydrocarbon.

15. The method of claim 4, the non-polar organic solvent is from about 1% to about 50% by volume of said composition.

16. The method of claim 4, wherein the polar organic solvent comprises at least one alcohol, ketone, or ether.

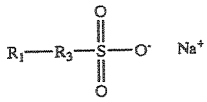
17. The method of claim 4, wherein the polar organic solvent is from about 1% to about 50% by volume of said composition.

18. The method of claim 4, wherein the surfactant comprises at least one cationic surfactant having the formula



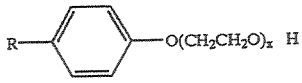
wherein R_1 is methyl, ethyl or propyl or isopropyl where n is 1 or 2; $R_2$is an alkyl from CH_3 to $C_{30}H_{61}$ or a benzyl group; and R_3 is (CH_2) where m is from 1 to 10, R_1 is $(OCH_2CH_2)_p$ where p is from 1 to 10.

19. The method of claim 4, wherein the surfactant comprises at least on anionic surfactant having the formula



wherein R_1 is C_6H_{13} to $C_{30}H_{61}$ and R_3 is CH_2 or a phenyl group.

20. The method of claim 4, wherein the surfactant surfactant comprises at least one non-ionic surfactant having the formula



wherein R is a linear or branched C1 to C10 alkyl group and X is from 5 to 40.

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